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**Progettazione e sintesi di nuovi antagonisti CB1 a struttura
pirazolica come potenziali radiofarmaci per la PET imaging**

CHIM08

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A mia madre

Abstract

Cannabinoid receptors are members of the large family of G-protein coupled receptors. Two types of cannabinoid receptor have been discovered: CB1 and CB2. CB1 receptors are localised predominantly in the brain whereas CB2 receptors are more abundant in peripheral nervous system cells. CB1 receptors have been related with a number of disorders, including depression, anxiety, stress, schizophrenia, chronic pain and obesity. For this reason, several cannabinoid ligands were developed as drug candidates. Among these ligands, a prominent position is occupied by SR141716 (Rimonabant), which is a pyrazole derivative with inverse agonist activity discovered by Sanofi-Synthelabo in 1994. This compound was marketed in Europe as an anti-obesity drug, but subsequently withdrawn due to its side-effects. Since the relationship between the CB1 receptors' functional modification, density and distribution, and the beginning of a pathological state is still not well understood, the development of radio-ligands suitable for in vivo PET (Positron Emission Tomography) functional imaging of CB1 receptors remains an important area of research in medicine and drug development. To date, a few radiotracers have been synthesised and tested in vivo, but most of them afforded unsatisfactory brain imaging results. A handful of radiolabelled CB1 PET ligands have also been submitted to clinical trials in humans. In this PhD Thesis the design, synthesis and characterization of three new classes of potential high-affinity CB1 ligands as candidate PET tracers is described.

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Scopo

In questo lavoro di tesi viene descritta la progettazione, la sintesi, la caratterizzazione e l'attività biologica di composti a potenziale attività antagonista dei recettori cannabinoidi 1 (CB1) allo scopo di individuare i candidati selettivi più promettenti da funzionalizzare successivamente con l'introduzione dei radioisotopi ^{18}F e ^{11}C e utilizzare con le apparecchiature di PET (tomografia ad emissione di positroni) imaging.

Le molecole radiomarcate dei CB1 potranno essere utilizzate:

- per valutarne la capacità di oltrepassare la barriera ematoencefalica e raggiungere il cervello, dove si trovano prevalentemente i recettori CB1;
- per studi dettagliati di imaging sui recettori CB1 del sistema nervoso centrale in modelli animali.

La prospettiva futura è quella di utilizzare questi strumenti molecolari come strumenti diagnostici nel cervello umano.

L'attività di ricerca è stata svolta in parte nei laboratori dell'azienda PharmaNess-Neuroscienze Scarl, presso il Parco Scientifico della Sardegna (Pula, CA) e in parte presso i laboratori dell'Institute of Medical Sciences dell'Università di Aberdeen. Il progetto di questa tesi rientra nel progetto FP7-PEOPLE-2009 IAPP - Marie Curie Action: "Industry-Academia Partnerships and Pathways" intitolato "Pet Brain. Mapping the brain with PET radiolabeled cannabinoid (CB1) ligands".

1 Introduzione

1.1 Cenni storici e classificazione botanica

La canapa (cannabis) è una pianta dai molteplici usi, e il suo impiego a scopo medico si perde nella notte dei tempi. La troviamo già citata nel più antico testo cinese sulle piante medicinali il Pen T'Sao Ching. Anche in India la cannabis è usata dalla medicina tradizionale fin dal secondo millennio a.c. Viene citata in antichi testi assiri, egizi e persiani, ed è ben conosciuta anche dalla medicina greco-romana.¹

Secondo la tassonomia ufficiale moderna, il genere *Cannabis* è incluso nella famiglia delle Cannabaceae dopo essere stato precedentemente inserito nelle Moraceae e successivamente nelle Urticaceae, viste le molte caratteristiche in comune con queste famiglie.

Nel 1753, Linneo parlò esclusivamente di *Cannabis sativa* L. l'indicazione di una *Cannabis indica* è dovuta a Lamarck e risale al 1783 ma oggi è considerata un errore di classificazione. Il binomio *Cannabis indica* e *Cannabis sativa* però si è mantenuto in letteratura farmacologica e tossicologica, creando così forte confusione.

Schultes, direttore del Museo Botanico di Harvard, nel 1974 espose la sua classificazione distinguendo la *Cannabis sativa*, *C. indica* e *C. ruderalis*.

Nel 1976 Small e Cronquist hanno proposto un diverso approccio, basando le differenze sulle proprietà della pianta. Il genere *Cannabis* avrebbe una sola specie (*C. sativa*) altamente variabile soprattutto per intervento dell'uomo. Identificando poi un gruppo del nord con maggiore contenuto di fibra e olio (*C. sativa* subs. *sativa*) e un gruppo del sud con maggior potenziale inebriante (*Cannabis sativa* subs. *indica*).²

Le prime pubblicazioni in riviste mediche sulla *Cannabis* risalgono al 1840 quando lo psichiatra J.J. Moreau condusse un esperimento clinico in seguito alla somministrazione di hashish per l'uomo, scoprì gli effetti del fumo di hashish e cominciò a studiarli al fine di comprendere il rapporto tra follia e sogni.³ Intorno alla fine del XIX secolo, dopo acetilazione di un estratto di hashish è stato

ottenuto il cannabinolo acetato. La sua struttura è stata individuata nel 1930, quando è stato isolato anche il cannabidiolo (CBD). Roger Adams e Alexander Todd pubblicarono numerosi documenti sulla Cannabis e scoprirono che alcuni composti triciclici sintetici avevano attività sui cani. Loewe nel 1950 riassunse il lavoro farmacologico effettuato su estratti di cannabis e di alcuni composti sintetici.⁴

Sono state distinte tre classi di cannabinoidi: naturali, endogeni e sintetici.

1.2 Cannabinoidi naturali (fitocannabinoidi)

La pianta Cannabis sativa contiene centinaia di sostanze a varia struttura chimica. Di queste, fino a oggi, sono stati identificati circa 66 composti appartenenti alla famiglia dei cannabinoidi, accomunati da una particolare struttura di 21 atomi di carbonio: derivano dalla combinazione di un elemento terpenico ad un fenolo di tipo chetidico, l'olivetolo, sono caratterizzati da una bassa polarità e solubilità in acqua (Fig. 1).⁵

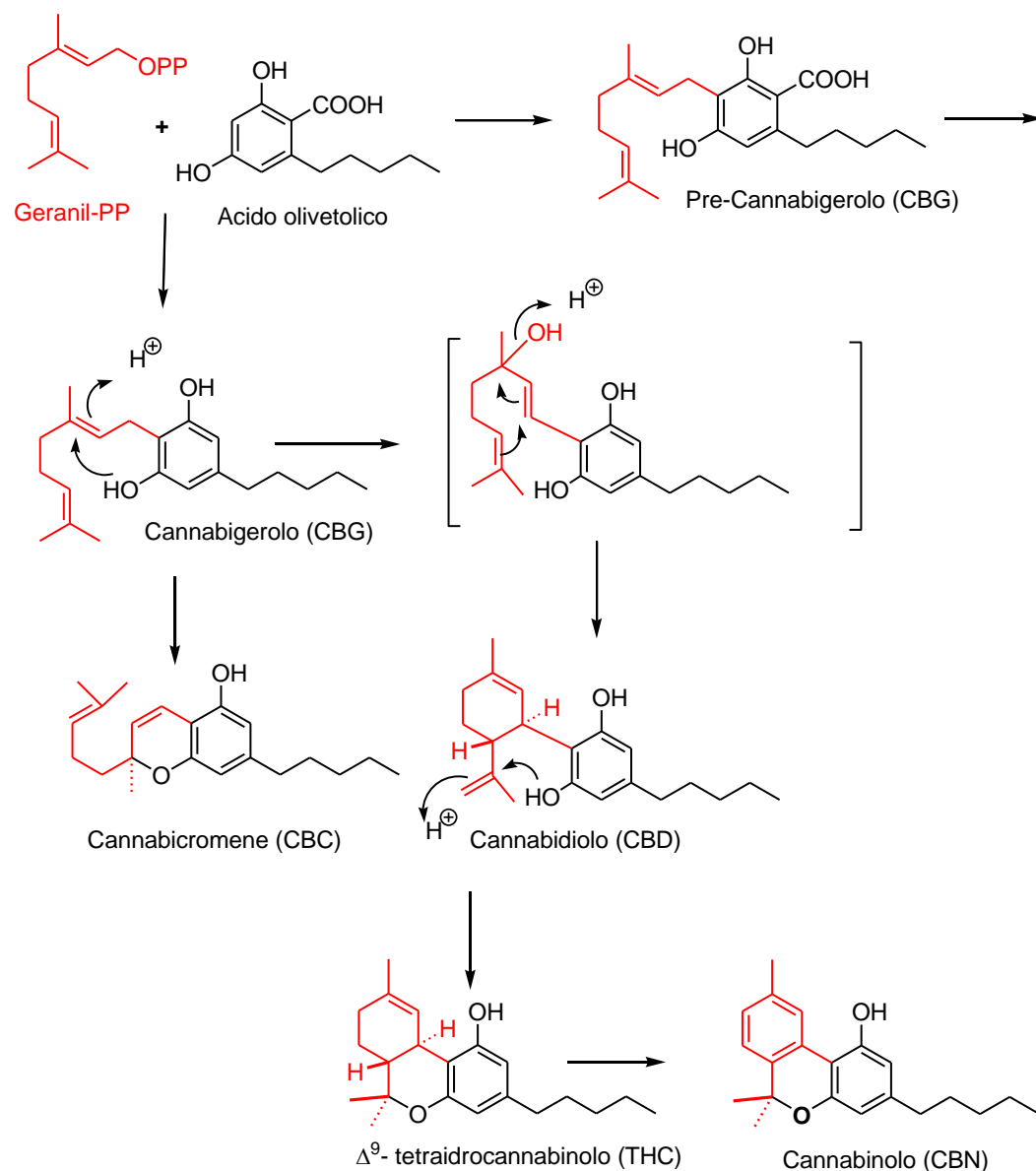


Figura 1: Alcuni composti cannabinoidi contenuti nella *Cannabis sativa* e indicazione dei precursori naturali.

Fitocannabinoidi sono presenti anche in diverse specie vegetali oltre alla *Cannabis*. Questi includono le N-alchilamidi che ritroviamo nell'*Echinacea purpurea*, *Echinacea angustifolia*, *Echinacea pallida*,^{6, 7} le N-alchildien-trienamidi nell'*Otanthus maritimus*,⁸ il falcarinolo nelle Apiaceae (es. *Daucus carota*),⁹ la rutamarina nella *Ruta graveolens* L.,¹⁰ e altri composti.^{11, 12}

Il Tetraidrocannabinolo (THC) è il componente psicoattivo principale della Cannabis. Il Δ^9 -THC e Δ^8 -THC, mimano l'azione dell'anandamide, un neurotrasmettitore prodotto naturalmente nel corpo. THC e gli altri cannabinoidi sono liposolubili, quindi possono accumularsi nel tessuto adiposo dal quale vengono lentamente rilasciati determinando un prolungamento dell'effetto del composto.

Il cannabidiolo non è psicoattivo così come il cannabichromene.

Piante di cannabis possono mostrare un'ampia diversità di quantità e del tipo di cannabinoidi che producono. Il dronabinolo (il trans-delta-9-tetraidrocannabinolo) e il nabilone vengono usati, in altri paesi, per trattare la nausea e vomito indotti da chemioterapia in persone in cui altri farmaci per il trattamento di questo tipo di nausea non sono più efficaci e il trattamento per la perdita di appetito e perdita di peso nelle persone affette da AIDS.¹³ Nel 2013 è entrato in commercio il Sativex, una miscela contenente due principi attivi: il THC e il CBD. Il farmaco viene utilizzato per il trattamento del dolore tumorale e del dolore neuropatico nei pazienti affetti da sclerosi multipla.¹⁴

1.3 Endocannabinoidi

L'identificazione del sistema endocannabinoide viene fatta risalire all'anno in cui venne identificato il Δ^9 THC (1964), il componente attivo della Cannabis.¹⁵ Ancora oggi gli studi continuano ad analizzare gli effetti di questo composto attivo sul nostro corpo. Inizialmente si pensava agisse come un semplice analgesico, in seguito si scoprì che il THC agiva su un recettore specifico. Solo in seguito vennero individuati due tipi di recettori (CB1 e CB2). La scoperta della presenza di questi recettori indusse alcuni ricercatori ad intuire che dei composti endogeni agissero sugli stessi recettori e li chiamarono endocannabinoidi. L'isolamento del primo di questi composti, l'anandamide, è stata fatta solo una trentina di anni fa.¹⁶

Tra gli endocannabinoidi (Fig.2) ci sono anche il 2-arachidonoil-glicerolo, il 2-arachidonilgliceril etere (noladin etere),¹⁷ e la N-arachidonoil-dopamina (NADA).¹⁸

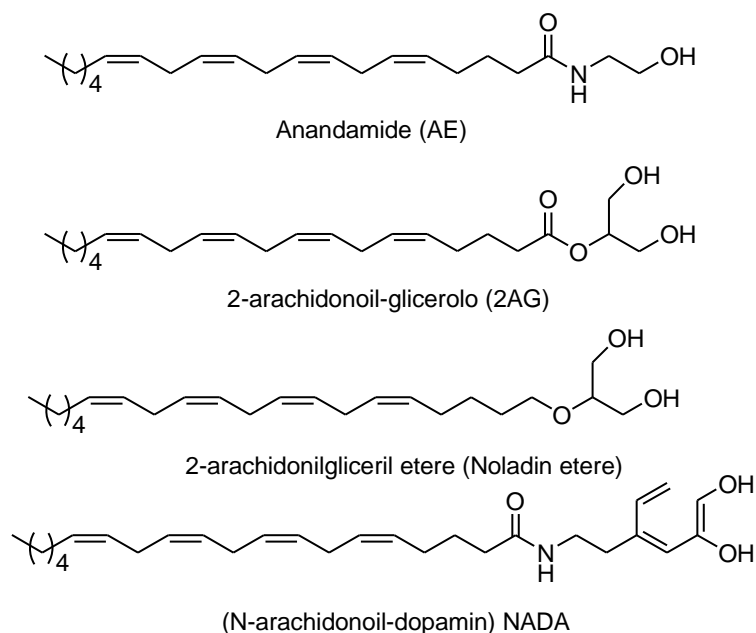
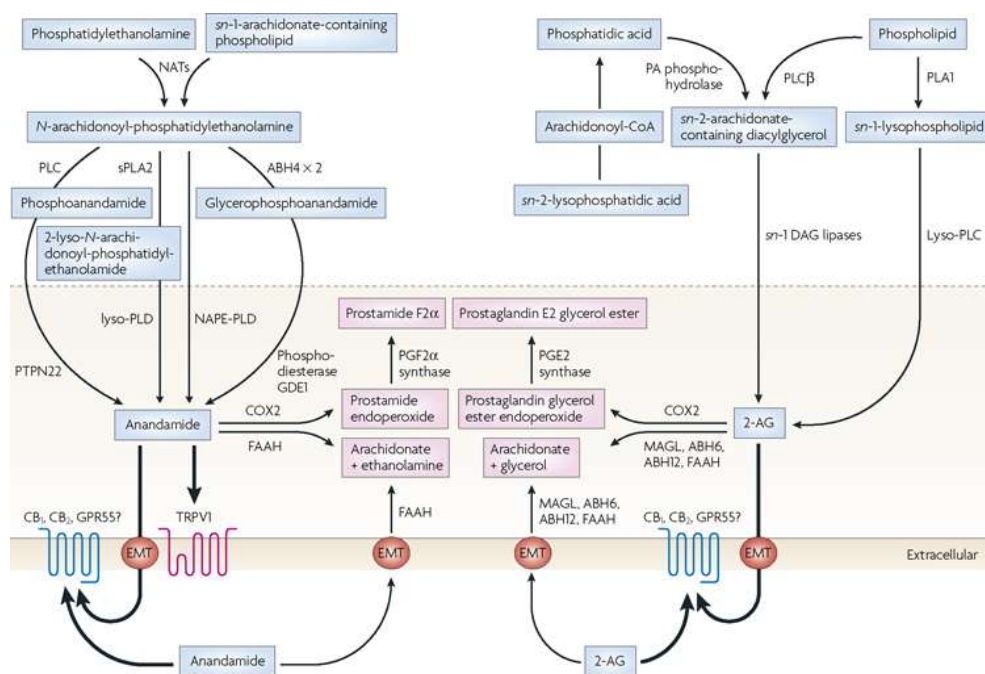


Figura 2. Alcuni composti Endocannabinoidi

Al contrario di altri mediatori chimici cerebrali questi composti non sono prodotti e immagazzinati nelle cellule nervose ma prodotti solo quando necessario, in seguito a stimoli cellulari.¹⁹ Questo comportamento, diverso rispetto alla maggior parte dei neurotrasmettitori, sembra legato alla natura lipofila che caratterizza questi ligandi: infatti le molecole fosfolipidiche di membrana servono per essi sia da precursori, sia da forma di deposito. In particolare l’N-arachidonilfosfatidiletanolamina (NAPE) è il precursore dell’anandamide, che subisce conversione ad opera della fosfolipasi D. L’anandamide è rimossa dai siti d’azione attraverso ricaptazione cellulare da parte di proteine carrier transmembranarie, proteine leganti associate alla membrana, e/o diffusione semplice. Per quanto riguarda la via catabolica l’anandamide subisce metabolizzazione intracellulare ad acido arachidonico ed etanolamina, catalizzata dall’idrolasi delle ammidi degli acidi grassi (FAAH), enzima che nei neuroni centrali è situato prevalentemente nelle superfici citosoliche delle cisterne del reticolo endoplasmatico liscio e nei mitocondri.

L'anandamide è metabolizzata anche ad opera di altri enzimi: PAA (palmitoylethanolamide-preferring Acid Amidase), ciclossigenasi-2, lipossigenasi e citocromo P450. Il 2-arachidonoilglicerolo viene sintetizzato a partire dal diacilglicerolo, per azione della diacilglicerolo (DAG) lipasi. Analogamente all'anandamide, il 2-AG viene rimosso dai siti d'azione attraverso dei meccanismi di ricaptazione cellulare e metabolizzato a livello intracellulare: la principale via di degradazione sembra essere quella della monoacilglicerolo (MAG) lipasi, che produce acido arachidonico e glicerolo.^{20, 21}



Nature Reviews | Drug Discovery

Figura 3. Biosintesi e l'inattivazione dell' anandamide e il 2-arachidonoilglicerolo (2AG) Le vie biosintetiche per anandamide e il 2-AG sono mostrati in blu, la via degradativa in rosa. ABH4/6/12 , α βidrolasi 4/6/12; CB1/2, recettori Cannabinoidi 1/2; COX2, cicloossigenasi 2; DAG, diacilglicerolo; EMT, trasportatore di membrana degli endocannabinoidi; FAAH , ammidolasi degli acidi grassi; GDE1, glicerofosfodiester fosfodiesterasi 1; GPR55, proteina G accoppiata al recettore 55; MAGL, monoacilglicerolo lipasi; NAPE-PLD, fosfodiesterasi selettiva della N- acilfosfatidiletanolamina; NAT, N-aciltransferasi; PA, acido fosfatidico; PLA1/2, fosfolipasi A1/2 ; PLC , fosfolipasi C; PLCβ, fosfolipasi Cβ; PLD , fosfolipasi D; TRPV1, sottotipo recettoriale vanilloide 1; PTPN22 , protein tirosina fosfatasi N22.²⁰

Il sistema endocannabinoide è coinvolto in un gran numero di processi fisiologici,^{22, 23} tra i quali:

- *Controllo motorio.* Gli endocannabinoidi e i loro recettori CB1 sono abbondantemente distribuiti nelle aree cerebrali deputate al controllo motorio coordinazione motoria e del movimento (per esempio, il cervelletto, i gangli della base, nello specifico, lo striato e la substantia nigra). Alcuni studi hanno evidenziato un'alterata funzione dei recettori CB1 a livello dei gangli della base sia in modelli animali che in uomini affetti da diverse patologie neurologiche. Gli effetti degli endocannabinoidi sull'attività motoria sono presumibilmente legati alla loro azione modulante sull'attività di diversi neurotrasmettitori coinvolti nel controllo motorio, quali dopamina, GABA e glutammato.²³

- *Memoria e l'apprendimento.* L'effetto peggiorativo della marijuana sulla memoria è ben riconosciuto, e si pensa che un ruolo chiave sia esercitato dai recettori cannabinoidi a livello dell'ippocampo. Infatti, l'ippocampo rappresenta una delle regioni cerebrali a maggiore densità dei recettori CB1. Quest'area è implicata nei processi di apprendimento e consolidazione mnemonica, e diversi studi hanno confermato severe alterazioni della memoria a breve termine in seguito all'uso della marijuana, che sono simili a quelle trovate in forme di demenza caratterizzate da degenerazione ippocampale, quale la malattia di Alzheimer.²⁴

Un possibile meccanismo ipotizzato è quello che gli endocannabinoidi inibiscono il rilascio di diversi neurotrasmettitori, quali GABA, glutammato e acetilcolina²⁵ e che la riduzione del rilascio di glutammato a livelli inferiori a quelli necessari per l'attivazione dei recettori della N-metil-D-aspartato (NMDA) sia responsabile della riduzione di memoria a breve termine osservata nelle intossicazioni da cannabis, essendo questi recettori implicati nella formazione della memoria.

- *Percezione del dolore.* Antinocicezione indotta dalla somministrazione di cannabinoidi esogeni è stato ampiamente riportato. Si pensa attualmente che gli endocannabinoidi partecipino in un sistema naturale di analgesia, mediato dai recettori CB1 a livello del tronco cerebrale e nel midollo spinale.^{26, 27} Infatti, in seguito a stimoli nocivi e la somministrazione di antagonisti del recettore CB1 si

osservano nel tronco cerebrale alti livelli di endocannabinoidi che determinano iperalgesia, suggerendo la presenza di un controllo tonico della nocicezione mediato dai recettori CB1.²⁸

- *Regolazione dell'equilibrio energetico e regolazione dell'appetito.* Il sistema endocannabinoide è coinvolto nel controllo dell'assunzione di cibo sia negli animali e nell'uomo attraverso una modulazione degli aspetti di gratificazione del comportamento alimentare. Inoltre, gli endocannabinoidi ipotalamici sembrano essere parte di circuiti neurali coinvolti negli omeostasi energetica della leptina.²⁹ Pertanto, alterazioni del potrebbero essere coinvolti nella fisiopatologia dei disturbi alimentari, ruolo degli endocannabinoidi nella regolazione dell'assunzione di alimenti e nel controllo del peso. Questo potrebbe essere mediato da un effetto inibitorio degli endocannabinoidi sull'ormone leptina, responsabile della soppressione dell'appetito.

- *Modulazione del sistema immunitario.* I cannabinoidi modulano una varietà di funzioni cellulari immunitarie. In particolare è stato dimostrato un loro ruolo nella modulazione dello sviluppo delle cellule T helper, chemiotassi, e lo sviluppo del tumore. Molti di questi effetti si verificano attraverso i meccanismi di modulazione delle citochine e di altri prodotti genici. Questo coinvolgimento potrebbe essere sfruttato in future terapie per malattie croniche e immunodeficienza.³⁰

- *Neuroprotezione.* I recettori cannabinoidi sono importanti nelle patologie neurodegenerative.³¹⁻³³



Figura 4. Le principali funzioni del sistema endocannabinoide²²

1.4 Cannabinoidi sintetici

I cannabinoidi sintetici sono un gruppo eterogeneo di molecole sintetizzate per sondare il sistema dei cannabinoidi endogeni o come potenziali farmaci. Sono stati sviluppati nel corso degli ultimi 40 anni come potenziali agenti farmaceutici, spesso destinati per il controllo del dolore. Tuttavia, è risultato difficile separare le proprietà desiderate dagli effetti psicoattivi indesiderati. Oggi con il termine cannabinoidi, ci si riferisce a ogni composto che interagisce con i recettori cannabinoidi: CB1 e CB2. di segnale.

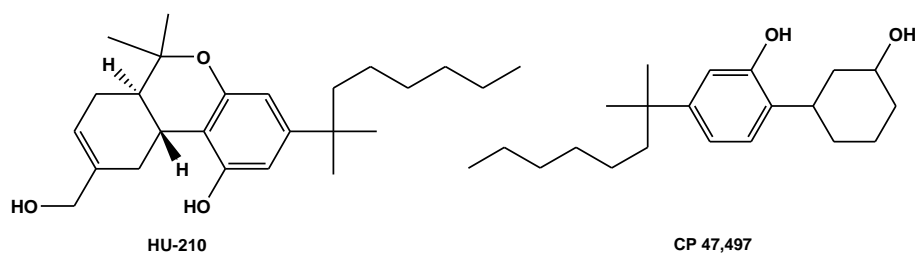
La maggior parte di tali molecole non è strutturalmente correlata ai cosiddetti “cannabinoidi classici” (per esempio i composti come il THC).

Agonisti non selettivi

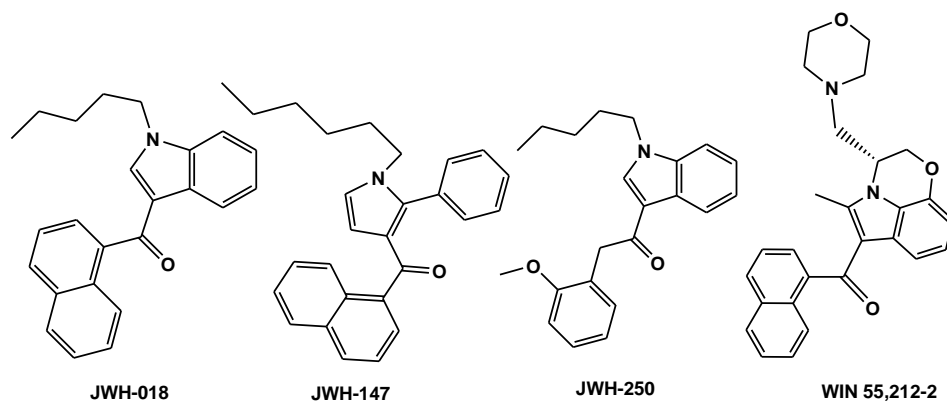
Molti di questi composti sono identificati come *Spice-drugs*: sono stati rilevati in miscele di erbe vendute su siti web. Sono composti che hanno effetti

simili al THC. Costituiscono un gruppo eterogeneo di sostanze, sebbene abbiano diverse caratteristiche in comune, tra cui la liposolubilità, la non polarità e siano costituiti da 22-26 atomi di carbonio. Caratteristica comune di questi cannabinoidi sintetici è la presenza di una catena laterale che conferisce attività alla molecola: un'attività ottimale richiede la presenza di più di quattro e fino a nove atomi di carbonio saturi.

I primi analoghi del THC furono sintetizzati nel 1980: il HU210 e alcuni cicloesilfenoli da Pfizer come analgesici, CP47,497. Il HU210 è circa 100 volte più potente THC. Questi composti che non sono in grado di discriminare tra i due recettori, per cui interagiscono modificando l'attività di entrambi i sottotipi recettoriali.



Tra le altre classi di cannabinoidi mimetici troviamo i naftoilindoli. Un esempio è il JWH-018, un potente agonista cannabinoide sintetico.³⁴ I naftilmetilindoli, i naftoilpirroli (es. JWH-147), i naftilmetilindani, i fenilacetilindoli (JWH-250) e aminoalchilindoli (WIN 55,212-2).³⁵⁻³⁷

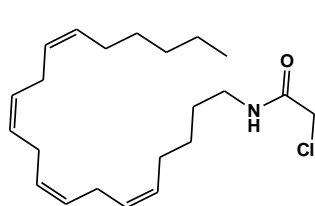


Questi composti sono in grado di: aumentare la frequenza cardiaca e la pressione sanguigna, creano un stato alterato di coscienza, leggera euforia e relax,

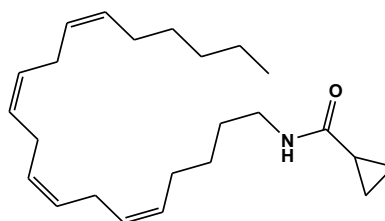
alterazioni percettive (distorsione temporale), intensificazione delle esperienze sensoriali, difficoltà di memoria a breve termine, riduzione dell'abilità motoria, aumento dei tempi di reazione. La struttura di questi nuovi cannabinoidi sintetici differisce da quella del THC, e probabilmente non rispondono in maniera positiva al test per i cannabinoidi negli immunodosaggi dei fluidi corporei.³⁵

Agonisti selettivi per i recettori CB1

In questa categoria rientrano composti analoghi dell'anandamide, ad esempio l'arachidonil-2-chloroethylamide (ACEA) e l'arachidonilciclopropilamide (ACPA), che presentano elevata selettività per il recettore CB1.³⁸ Queste molecole hanno la capacità di legarsi al recettore, di attivarlo, trasmettendo un segnale che viene, successivamente, decodificato dalla cellula.



ACEA



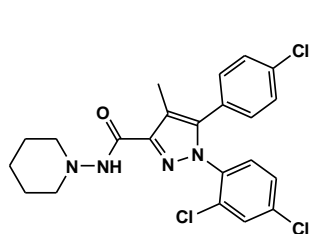
ACPA

Antagonisti/agonisti inversi dei CB1

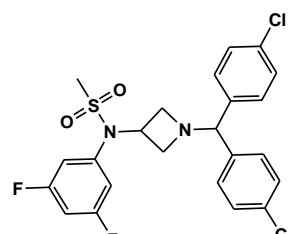
I primi tentativi di progettazione di antagonisti risalgono agli anni 80, l'idea iniziale era quella di modificare lo scaffold del THC. Fu sintetizzato il WIN 48,098, un aminoalchilindolo. Il composto però oltre a una scarsa attività presentava anche problemi di tossicità.³⁹ Lo scarso successo dei primi composti portò ad orientarsi verso molecole con struttura completamente diversa. Gli antagonisti del recettore CB1 sono stati sviluppati per essere utilizzati terapeuticamente per la riduzione del peso corporeo nei pazienti obesi o per ridurre i rischi associati (ad esempio dislipidemia e diabete di tipo 2) in pazienti in sovrappeso. Il primo di questi composti, Rimonabant, aveva con successo

completato quattro studi di Fase III e era stato immesso in commercio con il nome di Acomplia (K_i CB1= 2nM, K_i CB2 = 514 nM).^{40, 41} Attualmente è stato ritirato dal commercio nei paesi dell'Unione europea e in molti altri Paesi a causa di effetti collaterali correlati associati all'uso del farmaco in particolare a casi accertati di suicidio e depressione, comparsa di disturbi psichiatrici in molti soggetti trattati, mancata azione di rallentamento sulla progressione dell'aterosclerosi.⁴²

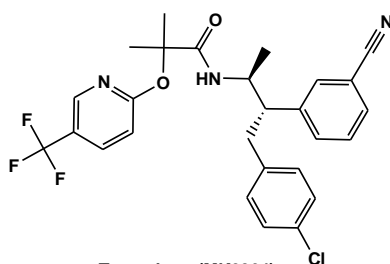
Hanno avuto lo stesso destino altri composti che avevano già superato la fase 3 degli studi clinici.



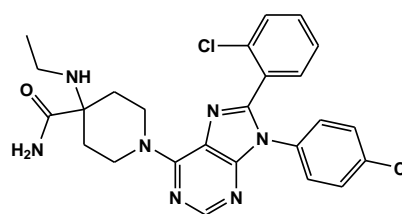
Rimonabant (SR141716, Acomplia)



Drinabant (AVE-1625)



Taranabant (MK0364)



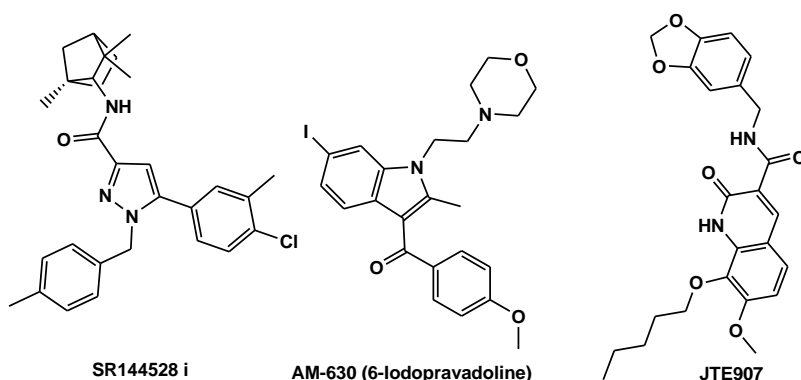
Otanabant (CP-945,598)

Antagonisti/agonisti inversi CB2

La prevalente distribuzione periferica dei CB2 suggerisce che lo sviluppo di ligandi CB2 non saranno inficiati da effetti collaterali psicoattivi legati cannabinoid.

Il primo composto sintetizzato dotato di una notevole selettività fu il composto SR 144528.⁴³ A questo studio seguirono altri studi di composti come il JTE907⁴⁴ e l'AM630⁴⁵. Queste molecole che si legano prevalentemente ai

recettori CB2 trovano applicazione in stati allergici come le dermatiti contatto e come antiinfiammatori.⁴⁶



Partendo inoltre dal presupposto che i CB2 sono localizzati prevalentemente nel sistema immunitario, tali recettori possano essere sfruttati per rallentare o addirittura arrestare la progressione della Sclerosi multipla, reputata una malattia autoimmune.⁴⁷

1.5 Recettori cannabinoidi

I recettori CB1 e CB2 appartengono ai recettori accoppiati a una proteina G, costituiti da sette segmenti transmembrana e con le porzioni C-terminale e N-terminale intracellulari.

Attraverso le G proteine di tipo inibitorio (Gi/o), i recettori CB1 trasducono il segnale agendo su diversi effettori intracellulari. In particolare i cannabinoidi inibiscono l'adenililciclasi, modulano alcuni canali ionici :inibiscono i canali del calcio di tipo N e P/Q mentre attivano i canali del potassio rettificanti entranti e stimolano la via delle MAP kinasi. Anche il recettore CB2 trasduce il segnale mediante G proteine, ma conosce effettori solo parzialmente sovrapponibili a quelli del CB1. In particolare, il recettore periferico non è in grado di modulare i canali ionici, mentre, come il recettore centrale, è negativamente accoppiato all'adenilciclasi ed è in grado di attivare le MAP kinasi.⁴⁸

Questi sottotipi recettoriali cannabinoidi differiscono per localizzazione e specificità di substrati. I CB1 risultano essere maggiormente espressi a livello

cerebrale come ad esempio a livello di ippocampo, gangli della base, cervelletto, corteccia. Una minore concentrazione è invece localizzata in altre aree per esempio nel tronco cerebrale dove sono localizzati i centri regolatori della respirazione e della funzione cardiaca e in altri tessuti periferici fra cui la vescica, i testicoli e l'ileo.⁴⁹ I recettori CB2 sono invece espressi principalmente a livello della milza, tonsille e nelle cellule immunitarie. Recentemente, sono stati identificati nel sistema nervoso centrale (cellule gliali e neuronali).⁵⁰

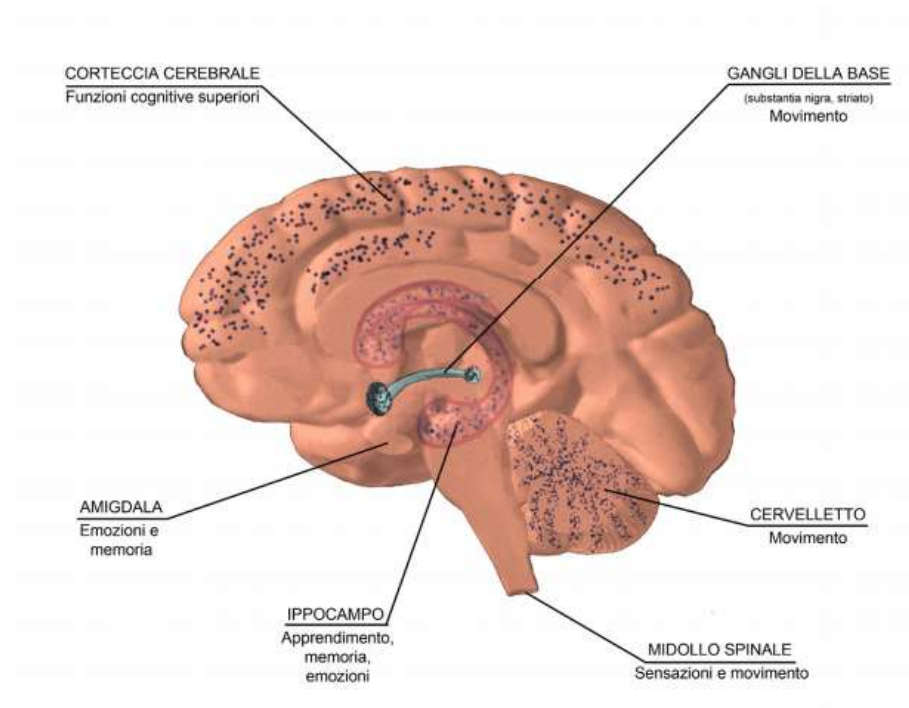


Figura 5. Distribuzione dei recettori CB1 nel cervello.

I recettori accoppiati alla proteina G possono oscillare tra stato attivo (R^*) e inattivi (R). Agonisti inversi possiedono maggiore affinità per la forma R , agonisti a R^* e agonisti neutri legano con affinità simile a entrambi gli stati del recettore. Pertanto, gli agonisti inversi diminuiscono la percentuale di R^* nella popolazione recettoriale, e ciò comporta una ridotta attivazione delle proteine G.⁵¹

Non esistono modelli cristallografici dei CB e gli studi computazionali vengono fatti considerando l'omologia con altri GPCR quali il recettore

adenosinico, A2A o la rodopsina.⁵² In figura è mostrato il recettore CB2 costruito per omologia e utilizzato per studi di docking su agonisti CB2.⁵³

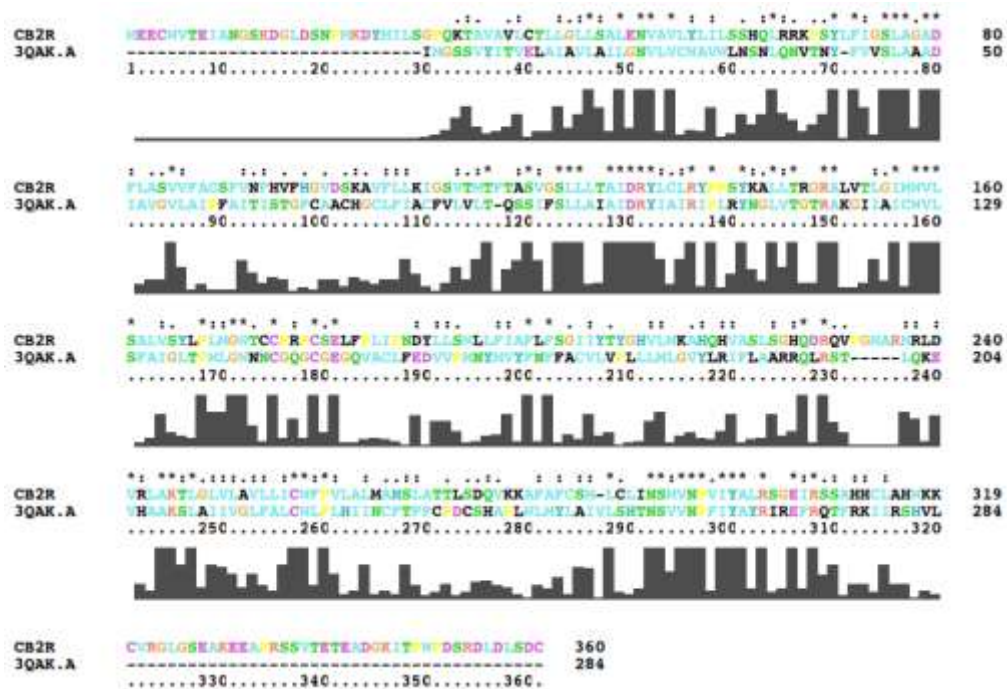


Figura 6. Allineamento delle sequenze dei recettori CB2 con la sequenza della recettore adenosinico 2A (pdb 3QAK).⁵³

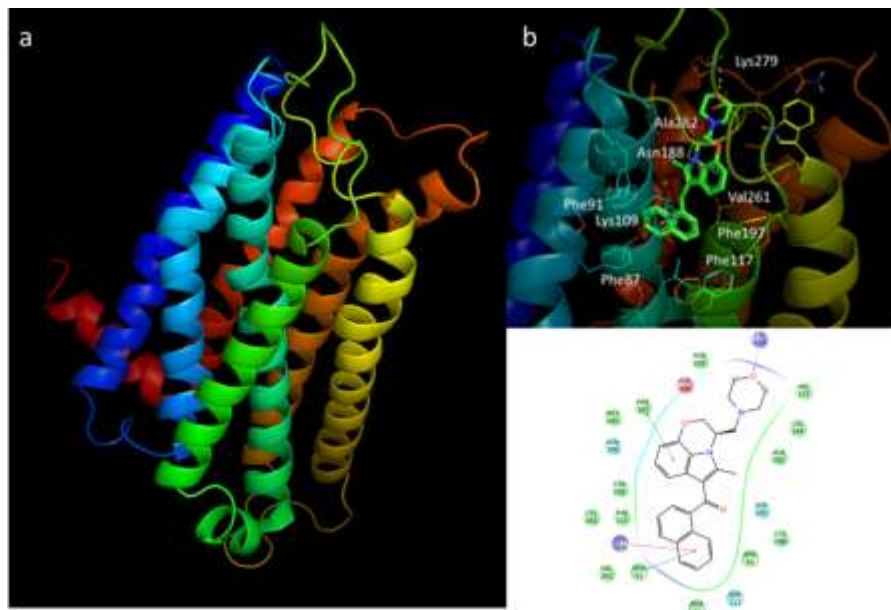


Figura 7. a) Recettore CB2 costruito per omologia con il recettore adenosinico; b) binding mode ipotizzato per il composto WIN 55,212-2.⁸

1.6 PET

La PET (positron emission tomography) è una tecnologia di imaging molecolare non invasiva che viene utilizzato per studiare e visualizzare fisiologia umana sfruttando il rilevamento di radiofarmaci ad emissione di positroni. Questa tecnica permette di ottenere informazioni in vivo su processi biologici a livello molecolare sia dal punto di vista qualitativo, visualizzando la biodistribuzione del tracciante specifico, sia dal punto di vista quantitativo poiché permette di stimare importanti parametri biologici.⁵⁴ Molte molecole biologiche possono essere marcate con radionuclidi, sorgenti di positroni, dando così origine ad un radiofarmaco. La PET mira all'ottenimento di immagini nitide con l'utilizzo di radioisotopi emettitori di positroni, particelle di massa pari agli elettroni e carica elettrica positiva.⁵⁵ L'isotopo decade, emettendo un positrone e genera una coppia di fotoni il cui segnale viene poi rivelato ed amplificato da opportuni sensori.

Dalla misurazione della posizione in cui i fotoni colpiscono il rivelatore, si possono ottenere le coordinate spaziali del punto da cui sono stati emessi, permettendo la determinazione dell'attività o dell'utilizzo chimico all'interno delle parti del corpo investigate. Solo alcuni radioisotopi possono essere utilizzati per la marcatura dei radiofarmaci PET, devono avere i requisiti adatti all'utilizzo in campo medico e clinico. Sono richieste, infatti, alcune caratteristiche specifiche per l'utilizzo in PET: 1) Il tempo di emivita ($t_{1/2}$) deve risultare adeguato al tipo di analisi eseguita; 2) Il radioisotopo deve essere prodotto in una forma chimica che ne permetta facilmente l'incorporazione in una molecola; 3) Il decadimento deve avvenire quasi interamente per positroni per garantire una buona qualità delle immagini PET registrate dai rivelatori nel corso dell'esame. Carbonio-11 ($t_{1/2}=20\text{min}$), Azoto-13 ($t_{1/2}=15\text{min}$), Ossigeno-15 ($t_{1/2}=2\text{min}$) e Fluoro-18 ($t_{1/2}=109.8\text{min}$) sono i più comuni radionuclidi utilizzati per la marcatura dei radiofarmaci PET, anche se negli ultimi anni stanno trovando spazio nel campo dell'Imaging Molecolare altri radioisotopi quali il Rame-64, lo Iodio-124, il

Bromo-76, il Gallio- 68. Tra tutti, comunque, il Fluoro-18 è indubbiamente la scelta più vantaggiosa per le reazioni di marcatura, in quanto si tratta di un radioisotopo dotato di requisiti ideali per la PET per il tempo di emivita, che garantisce studi a cinetica più lunga, essendo caratterizzato da una bassa energia del positrone emesso (0.693 MeV), che permette una migliore risoluzione delle immagini PET.⁵⁶

2 Progettazione

2.1 Base scientifica

Il sistema endocannabinoide è coinvolto in molteplici meccanismi e rappresenta una delle nuove frontiere tra i target farmacologici ed è per questo motivo che molti ligandi cannabinoidi sono stati sintetizzati. Tra questi ligandi, un posto di rilievo è occupato da SR141716 (Rimonabant). Un derivato pirazolico agonista inverso scoperto da Sanofi-Synthelabo (ora Sanofi-Aventis), nel 1994, commercializzato come un farmaco anti-obesità, ma successivamente ritirato dal mercato a causa dei suoi effetti collaterali, grave depressione e pensieri suicidi.^{40,}

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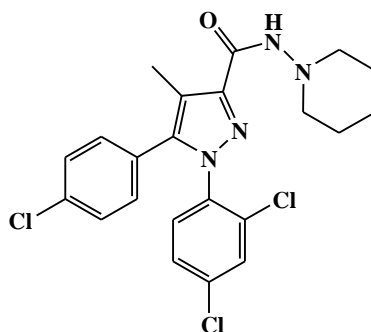


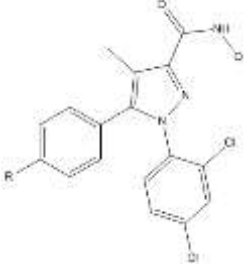
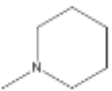
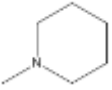
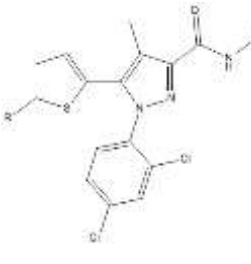
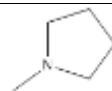
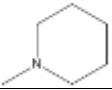

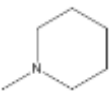
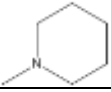
Figura 8. SR141716A (Rimonabant).

Sin da quando il Rimonabant fu identificato nel 1994 come il più potente e selettivo antagonista CB1, una grande varietà di composti analoghi sono stati sintetizzati negli anni inserendo modificazioni strutturali di vario genere. Prevalentemente le modifiche hanno riguardato l'anello pirazolico centrale con altri anelli eterociclici, nel tentativo di ottenere prodotti che mostrassero equivalenti caratteristiche di attività e selettività.^{57, 58} Nel brevetto US7659407B2⁵⁹ si è evidenziato come composti diarilpirazolici bioisosteri del Rimonabant, caratterizzati dalla sostituzione del fenile sul C5 del pirazolo con un anello tiofenico, tra i quali ricordiamo l'*N*-piperidinil-5-(cloro-tiofen-2-il)-1-(2',4'-diclorofenil)-4-metil-1*H*-pirazol-3-carbossammide, più nota come

NESS006A (Composto D), abbiano mostrato una significativa affinità e selettività nei confronti dei recettori CB1 (Tabella 1).⁵⁷⁻⁶¹

Alcuni di questi derivati sono stati testati, con risultati incoraggianti, nella cura dell'obesità.⁶⁰

Tabella 1 Affinità Recettori Cannabinoidi del Rimonabant e del NESS006A espressi in nM

		R	Q	K _i CB1	K _i CB2	K _i CB2/K _i CB1
	A	Cl		11.5	1643	142.8
	B	H		202	-	-
	C	Cl		63.0	699	11.1
	D	Cl		35.0	476	13.6
	E	Cl		5.5	70	12.7
	F	Br		17.4	781	44.9
	G	Ph		22.3	1610	72.2

Gli studi computazionali sul Rimonabant, e il confronto con la vastissima quantità di prodotti analoghi, hanno evidenziato quali siano le caratteristiche chimiche della struttura utili ai fini dell'attività e selettività di questi composti:

➤ la presenza del gruppo carbossamidico legato al C3 dell'anello pirazolico è indispensabile ai fini dell'interazione tra ligando e recettore. Infatti, l'ossigeno carbonilico forma un ponte idrogeno con il residuo di Lys192, mentre l'ammina ciclica viene ospitata in una tasca lipofila delimitata da residui idrofobici.⁶²

➤ La sostituzione in posizione N1 dell'anello pirazolico con il 2,4-diclorofenile stabilizza il complesso con il sito recettoriale.⁶³ Sebbene sembri che tale sostituito sia quello ottimale, la sua sostituzione con un gruppo 1-(5-isotiocianato)-pentile diminuisce solamente di un fattore 4 l'affinità nei confronti dei CB1.⁶²

➤ L'eliminazione della funzione ammidica ed una sua sostituzione con una chetonica, alcolica o eterea determina una considerevole diminuzione dell'affinità di binding nei confronti dei CB1.⁶⁴

➤ La disostituzione dell'azoto ammidico del Rimonabant diminuisce l'affinità verso i CB1.⁶⁵

➤ L'omologazione dell'anello piperidina sulla funzione carbossilica in C3 dell'anello pirazolico del Rimonabant, con la pirrolidina o la omopiperidina, così come la sostituzione con un cicloesile, non influisce sull'affinità CB1.^{64, 65}

➤ I composti recanti un metile, bromo o iodio in posizione 4 dell'anello pirazolico conservano la potenza della molecola madre, mentre la virtuale demetilazione nella stessa posizione risulta in una diminuzione di 12 ordini di grandezza nell'attività CB1.⁶⁴

➤ La sostituzione del 4-cloro sull'anello fenilico in posizione 5 dell'anello pirazolico con altro alogeno o gruppo alchilico non influisce sull'affinità di binding.⁶⁶ Invece la sostituzione con gruppi nitro o amminici, oppure lo spostamento dalla posizione 4-(para) alla posizione 2-(orto) del gruppo fenilico dà luogo a ligandi CB1 poco efficaci, mentre la sostituzione dell'anello aromatico con gruppi alifatici annulla l'affinità per i recettori CB.⁶⁵

Una combinazione di studi di mutagenesi e computazionali ha permesso di individuare come possibile modalità di binding quella riportata nella Figura 8 in cui i due anelli aromatici in posizioni 1 e 5 dell'anello pirazolo interagiscono positivamente con i residui Trp279/Phe200/Trp356 e Tyr275/Trp255/Phe278, rispettivamente, e allo stesso modo l'aminopiperidina con la cavità costituita da Val196/Phe170/Leu387 e Met384. Inoltre, il legame idrogeno tra l'ossigeno ammidico del ligando del recettore ed il residuo Lys192 svolge un ruolo cruciale nel legame, favorendo l'agonismo inverso del Rimonabant.^{67, 68}

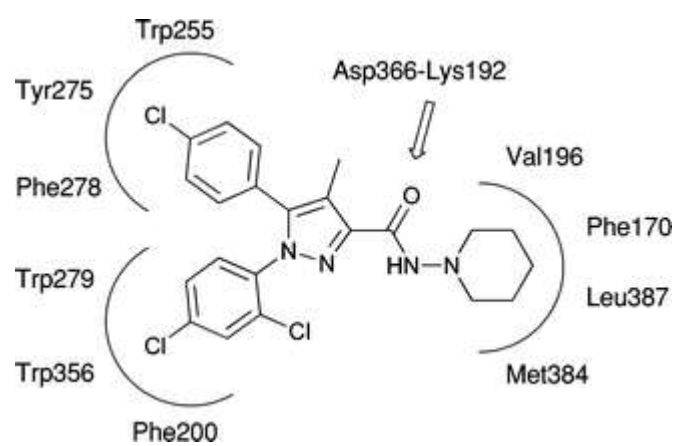


Figura 8. Interazioni tra Rimonabant e CB1

3 Sintesi e risultati

Derivati pirazolici della serie 1

Tenendo conto degli studi precedenti abbiamo deciso di sostituire la porzione carbonilaminopiperidinica in posizione 3 con una funzione 4-fluorobutil-(1,2,3-triazolica) in quanto gli atomi di azoto del gruppo triazolico sp² agiscono come accettori di legame idrogeno con Lys192.⁶⁹ Inoltre 1,2,3 – triazolo è in grado di essere funzionalizzato con un gruppo N-(4-fluorobutil), che potrebbe essere facilmente radiomarcato con ¹⁸F, quindi utilizzato come candidato tracciante PET. Infine questa porzione lipofila può essere ospitata nella tasca formata dai residui Val196/Phe170/Leu387/Met38.⁷⁰⁻⁷²;

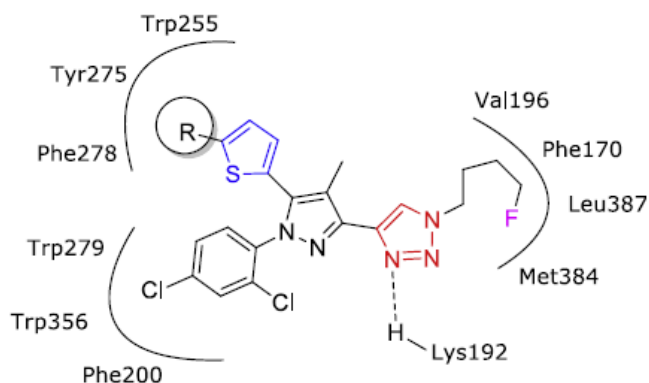
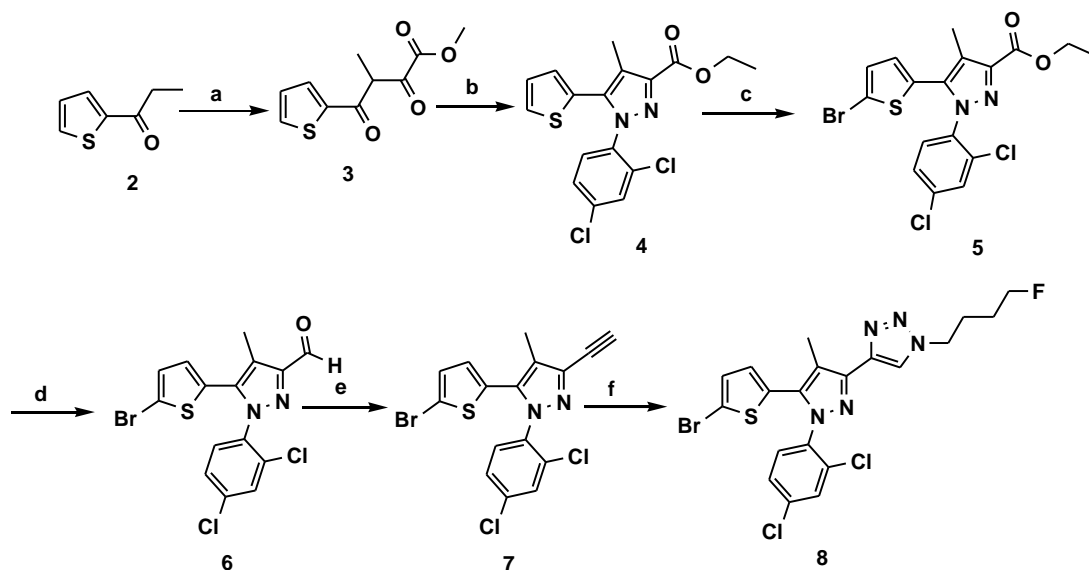


Figura 9: Ipotetica modalità di legame della serie sintetizzata, ottenuta per confronto con il Rimonabant (Fig8).

Sintesi

Lo schema sintetico applicato riprende la procedura classica utilizzata per la preparazione dei composti del NESS006A.⁵⁹



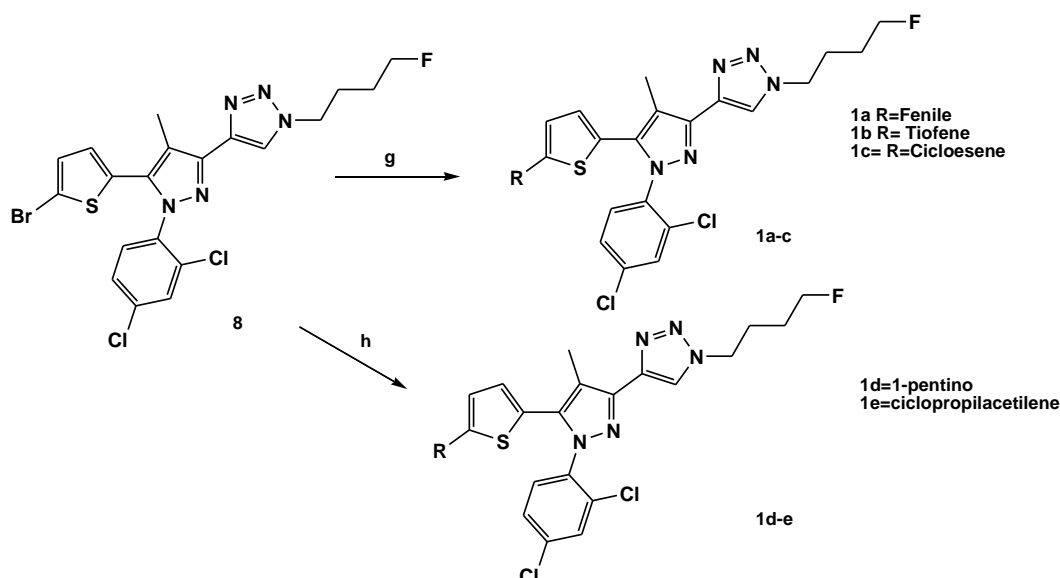
Schema 1: Reagenti e condizioni: a) dietilossalato, EtONa/EtOH, N₂, 20 ore R.T.; b) 2,4-diclorofenilidrazina, EtOH, refluxo 12 ore; c) NBS, CH₃CN, 0°C → T.A., 16 ore; d) DIBAL-H, DCM, -78-8°C, 4h; e) dimetil-1-diazo-2-ossopropilfosfonato, K₂CO₃, MeOH, R.T., 12h; f) 1-azido-4-fluorobutano, CuI, ascorbato di sodio, tert-BuOH/H₂O, R.T., 12h.

L' α - γ dichetoestere **3** è stato ottenuto mediante condensazione di Claisen, aggiungendo il chetone **2** ad una miscela di dietil ossalato e etossido di sodio in etanolo a temperatura ambiente (Schema 1). La chiusura dell'anello pirazolico viene eseguita tramite addizione della 2,4-diclorofenilidrazina in etanolo a temperatura ambiente per 24 h.⁶¹ La bromurazione regioselectiva dell'estere è stata effettuata usando NBS (N-Bromosuccinimide) in acetonitrile per ottenere il corrispondente bromurato.^{57, 73}

La riduzione dell'estere ad aldeide **6** è stata ottenuta utilizzando DIBAL-H (diisobutilalluminio idruro) a bassa temperatura seguita da *quench* con metanolo. La soluzione risultante di aldeide viene ulteriormente diluita con metanolo, trattata con K₂CO₃ e il reattivo Bestmann-Ohira; si è determinato così la conversione in alchino **7** con una moderata resa (55 %).⁷⁴ Il composto **8** è stato

ottenuto per reazione dell'opportuna azide e l'alchino **7** a temperatura ambiente in tert-butanolo/H₂O.

I composti **1a–c** sono stati ottenuti mediante reazione di Suzuki - Miyaura⁷⁵ catalizzata da palladio utilizzando il rispettivo acido boronico disponibile in commercio, mentre composti **1d–e** sono stati ottenuti in condizioni Sonogashira,⁷⁶ in presenza di tetrakis (trifenilfosfina) palladio, DIPEA (N,N-Diisopropiletilamina) e utilizzando il corrispondente alchino.



Schema 2 Reagenti e condizioni: g) DME (1,2-dimetossietano), Pd(PPh₃)₄, acido boronico, Na₂CO₃, ref. 20h; h) Pd(PPh₃)₄, alchino, DIPEA, CuI 80°C, 20h.

Test Biologici

Nella **Tabella 2** sono riportate le affinità dei composti nei confronti dei recettori CB1 e CB2, determinate mediante *radioreceptor binding* in accordo con le procedure precedentemente riportate in letteratura.^{77, 78}

Il composto **1d** si distingue per la sua elevata affinità CB1, che è paragonabile a quello visualizzato da Rimonabant (SR141716). Inoltre, **1d** ha mostrato discreta CB1/CB2 selettività (K_i CB2/K_i CB1 = 35.5). Tutti gli altri composti mostrano una K_i su CB1 un ordine di grandezza superiore a quella di **1d**, e bassa a moderata CB1/CB2 selettività. Composto **1d** è quindi il candidato più

promettente per un ulteriore sviluppo, compreso il suo possibile uso come tracciante PET per l'imaging.

Tabella 2. affinità dei composti nei confronti dei recettori CB1 e CB2.

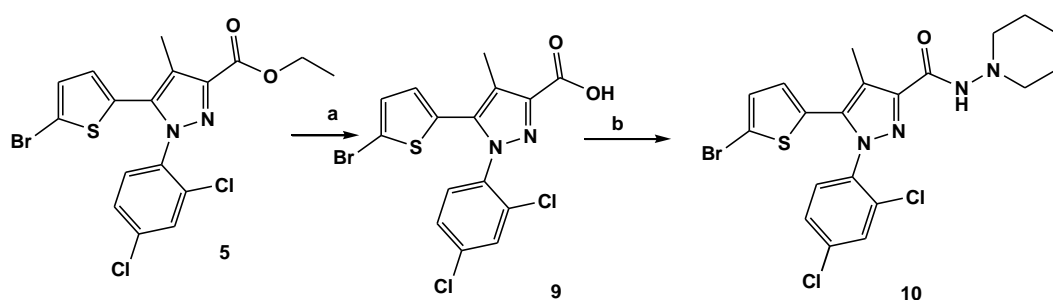
Composto	hCB1 Cellule ovariche di criceto(CHO)	hCB2 Cellule ovariche di criceto(CHO)
	Ki ^{a,d} (95% CL ^b)	Ki ^{a,d} (95% CL ^b)
1a	200 (94.9–420)	2.36 x 10 ³ (641–8.70 x 10 ³)
1b	353 (103–1.20 x 10 ³)	1.70 x 10 ³ (953–3.02 x 10 ³)
1c	119 (40.2–353) ^f	471 (95.1–2.33 x 10 ³)
1d	23.4 (6.80–80.0)	830 (281–2.45 x 10 ³)
1e	164 (32.5–825)	n.a. ^c
8	312 (113–862)	1.02 x 10 ³ (603–1.72 x 10 ³)
SR141716	18.7 (11.1–31.4) ^e	1.40 x 10 ³ (500–3.70 x 10 ³) ^g
^a nM. ^b CL, limite di confidenza. ^c impossibile raggiungere il plateau. ^d n = 4, se non indicato diversamente. ^e n = 14. ^f n = 12. ^g n = 2.		

Conclusioni

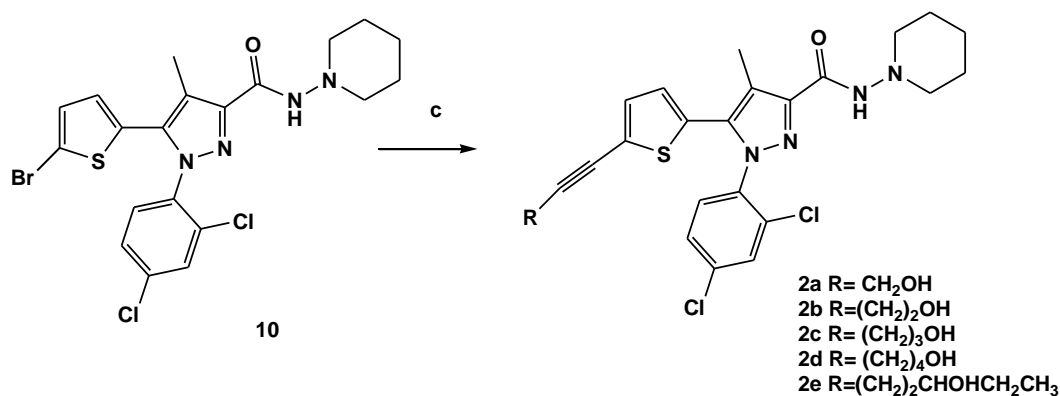
La sostituzione del gruppo carbonilico con un gruppo 1,2,3 - triazolico con funzione 4–fluorobutilica ha prodotto una nuova classe di ligandi del recettore CB1 con affinità nanomolare per il recettore CB1. Ciò può essere spiegato dalla capacità dell'anello 1,2,3 -triazolo di comportarsi come accettore di legame idrogeno con residuo di Lys192 del recettore CB1. In particolare la molecola **1d** è un promettente candidato per la radiomarcatura [18F] e quindi per l'impiego negli studi del recettore CB1 con utilizzo della PET Imaging,.

Derivati pirazolici della serie 2

Un'altra classe di composti sono stati sintetizzati con lo scopo di aumentare la lipofilità dei ligandi. In particolare è stato funzionalizzato il composto 5-(5-bromotiofen-2-il)-1-(2,4-diclorofenil)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (**10**), con un gruppo idrossialchilialcol che potrebbe essere facilmente radiomarcato con ^{18}F , quindi utilizzato come candidato PET tracciante.



Schema 3 a) KOH, MeOH, ref. 12h; b) HOBt(1-idrossibenzotriazolo idrato)(aq), EDC (N-(3-Dimetilaminopropil)-N-etilcarbodiimide)*HCl, CH_2Cl_2 1/2h R.T. 1-aminopiperidina, 20h R.T.;



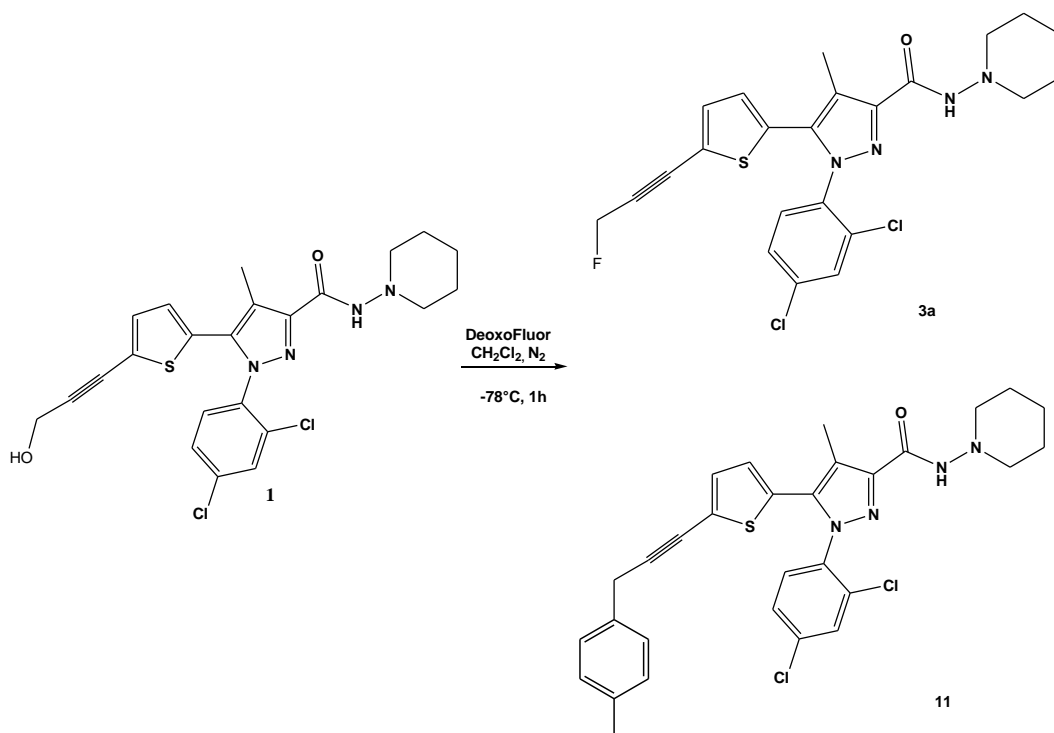
Schema 4: c $[\text{PPh}_3]_4\text{Pd}$, alchil-alcol DIPEA, 20' 40°C , CuI, 20h R.T.

Lo schema sintetico riprende quello della serie precedente. La N-piperidinil-carbossamide **10** è stata preparata a partire dall'estere (**5**) mediante idrolisi basica (**9**), seguita da reazione di *coupling* tra l'acido attivato e la 1-aminopiperidina.

Sono stati ottenuti cinque 1-(2,4-diclorofenil)-5-(5-(3-idrossialchinil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamidi (Schema 4, composti **2a-e**).

Questi sono stati successivamente deidrofluorurati ottenendo le 1-(2,4-diclorofenil)-5-(5-(fluoroalchinil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamidi (Schemi 5-6-7-8, composti **3a-e**).

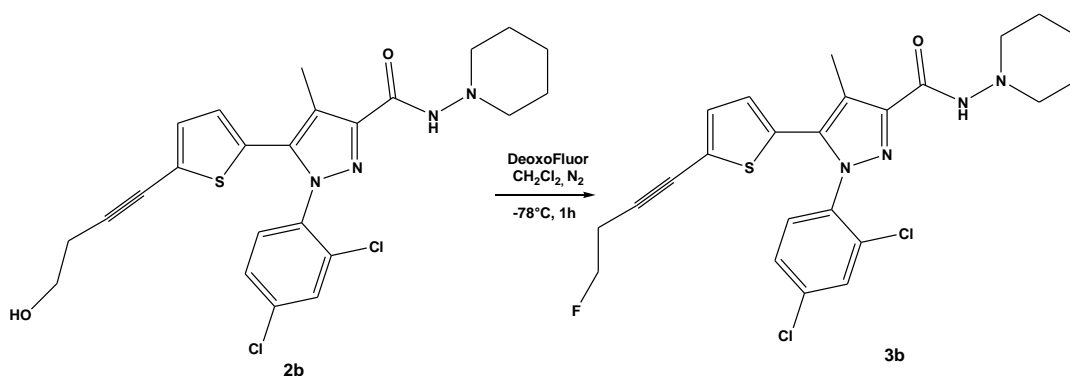
La deidrofluorurazione è stata inizialmente effettuata in diverse condizioni sia con DAST (Dietilaminosulfurtrifluoride) che con soluzione al 50% di DeoxoFluor in THF, ma nessun prodotto è stato ottenuto. La reazione quindi è stata condotta utilizzando una soluzione al 50% di Deoxofluor in toluene, che ha portato ai prodotti desiderati (con rese molto basse) e anche alcuni prodotti non desiderati che sono stati identificati (**11,12,13**)



Schema 5: Deidrofluorurazione di 1-(2,4-diclorofenil)-5-(5-(3-idrossiprop-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazolo-3-carbossamide

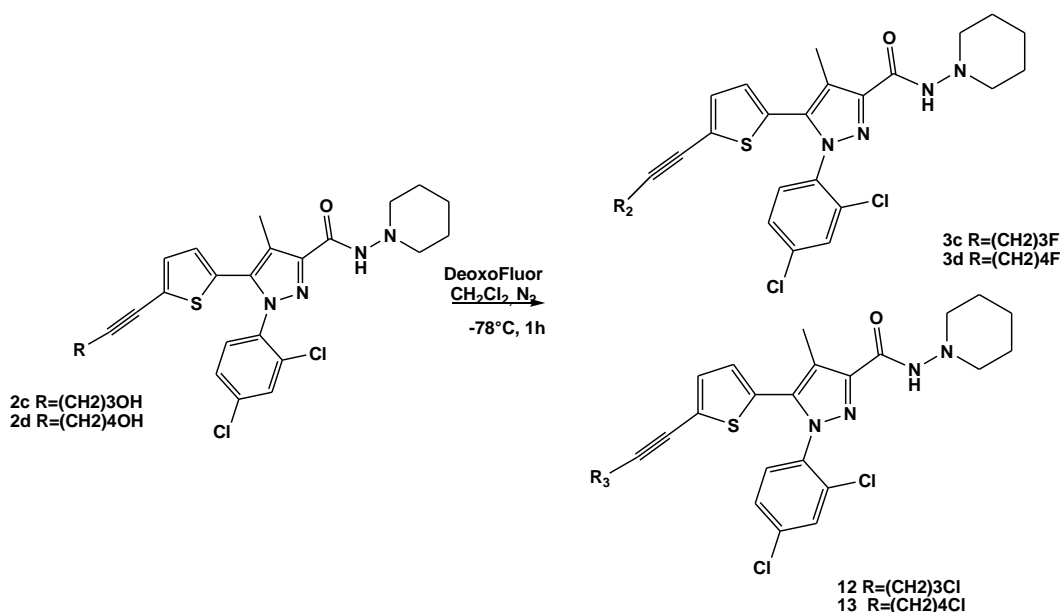
La reazione tra 1-(2,4-diclorofenil)-5-(5-(3-idrossiprop-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (**2a**), con la soluzione al 50% di DeoxoFluor in toluene, ha dato due prodotti principali: il composto

fluorurato desiderato **3a** e una notevole quantità di 1-(2,4-diclorofenil)-4-metil-N-(piperidin-1-il)-5-(5-(3-p-tolilprop-1-inil)tiofen-2-il)-1H-pirazol-3-carbossamide (**11**). Il processo di eliminazione della reazione tra alcol propargilico e DeoxoFluor provoca la formazione di un catione propargile intermedio, che reagisce con il toluene contenuto nel solvente attraverso una propargilazione, simile a quello che è stato precedentemente descritto da Li e Wang.⁷⁹



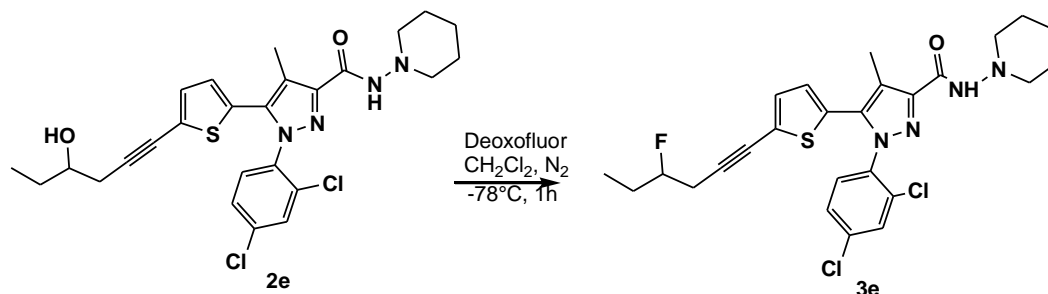
Schema 6: Deidrofluorurazione di 1-(2,4-diclorofenil)-5-(5-(4-idrossibut-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide

Particolarmente interessanti sono i due prodotti ottenuti dalle reazioni deidrofluorurazione con DeoxoFluor in toluene dei due alcool intermedi **2c** e **2d**. Sono stati ottenuti due prodotti alogenati in ciascuna reazione: il composto fluorurato atteso e quello corrispondente clorurato. Le cause di questo comportamento insolito non sono chiare. Potremmo ipotizzare che si abbia una sostituzione nucleofila in parallelo attraverso entrambi i percorsi SN1 e SN2. Il fluoro-composto potrebbe essere il risultato del meccanismo SN2, mentre un processo SN1 competitiva potrebbe portare alla formazione di un catione di vinilico ciclico stabilizzato dalla bassa temperatura che potrebbe essere intercettato dal diclorometano, un solvente relativamente inerte, formando i derivati clorurati inaspettati. Questa ipotesi spiegherebbe anche il motivo per cui solo i due prodotti con catena lineare abbastanza hanno portato a tali prodotti.⁷⁵



Schema 7: Deidrofluorazione di 1-(2,4-diclorofenil)-5-(5-(5-idrossipent-1-ynil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide.

Infine la deidrofluorurazione di **2e**, ha dato, come previsto, una miscela dei prodotti, ma il prodotto **3e** derivato è stato ottenuto con una buona resa .

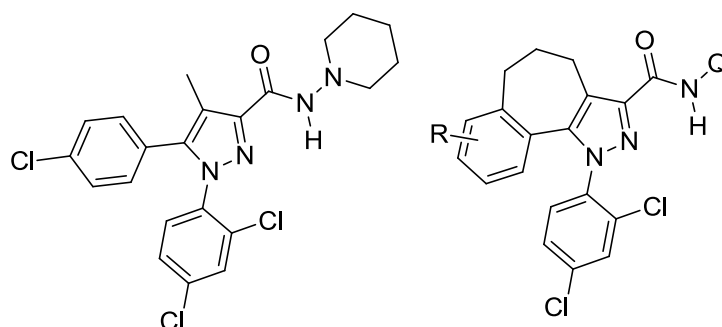


Schema 8 Deidrofluorurazione di 1-(2,4-diclorofenil)-5-(5-(4-idrossies-1-ynil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide.

L'attività biologica dei derivati fluorurati ottenuti non è ancora stato studiato.

Derivati pirazolici della serie 3

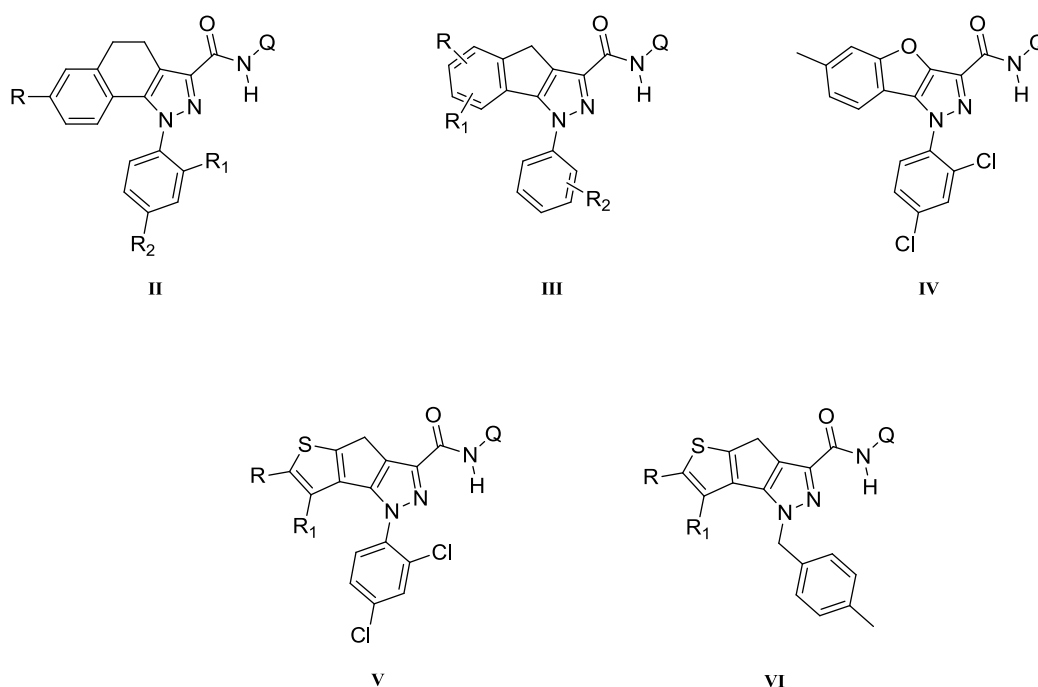
In letteratura sono presenti diversi studi SAR su varie serie di condensati cannabinoidi basati su pirazoli triciclici (serie I-VI) evidenziando parametri strutturali importanti nella modulazione di CB1 CB2 (Figura 10-11).⁸⁰⁻⁸⁵



rimonabant

I

Figura 10. Rimonabant and una serie di composti analoghi ma rigida dovuta alla presenza del pirazolo inserito in un nucleo condensato (**I**).



II

III

IV

V

VI

Figura 11. Altre serie di altri pirazoli triciclici con buona attività su CB₁ e CB₂.

Il composto NESS0327 (Fig. 12), era stato pubblicato dal nostro gruppo.⁸⁶ L'affinità mostrata verso i CB₁ è pari a $K_i = 0,00035 \pm 0,000005$ nM e sui CB₂ $K_i = 21 \pm 0.5$ nM. Il composto si comportava come un antagonista CB₁ in entrambe le prove: in vivo (topo in cui l'antinocicezione era stata indotta da WIN 55,212-2) e in ex vivo saggi (organi isolati del topo). Altri due gruppi hanno descritto la sintesi e la valutazione biologica del NESS0327 mostrando un'affinità per i CB₁ nettamente diversi da quello precedentemente riportato dal nostro gruppo. Il

composto è stato sintetizzato sia secondo la stessa procedura adottata dal nostro gruppo⁸⁷ sia attraverso un protocollo diverso.⁸⁸

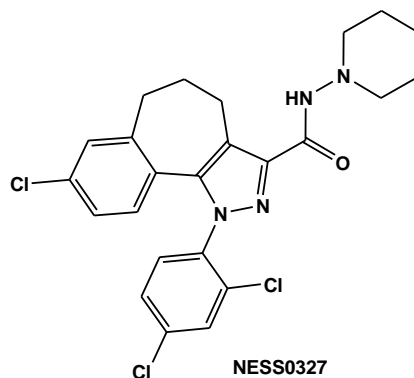


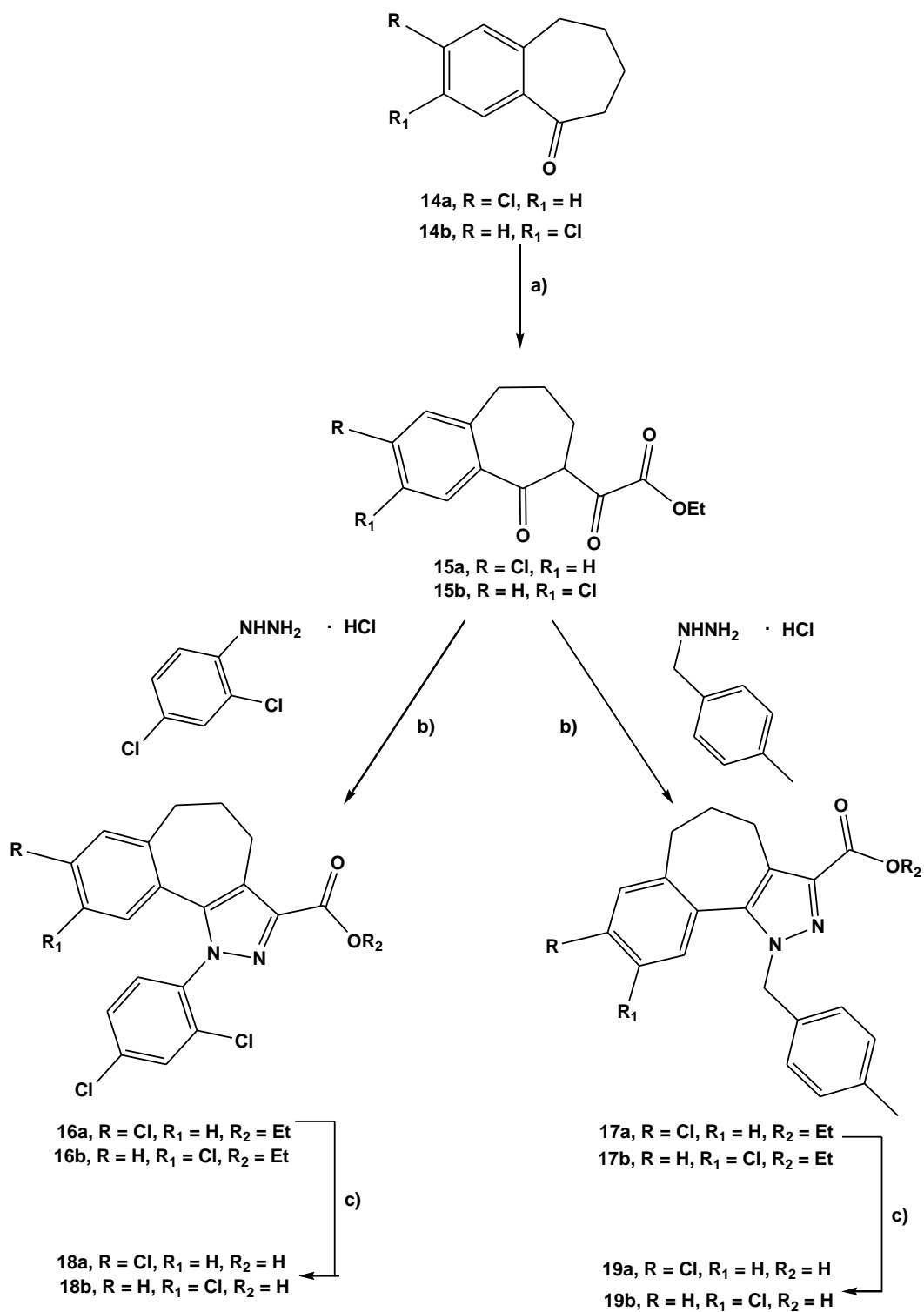
Figura12. NESS0327

Per approfondire la discrepanza tra questi dati, abbiamo risintetizzato il NESS0327. Due differenti metodi sono stati utilizzati così come riportato nel precedente articolo^{86, 89} e in un secondo lavoro.⁸⁸ I due prodotti finali sono stati poi confrontati sia mediante analisi chimica e sia mediante misurazione dell'affinità.

Sintesi

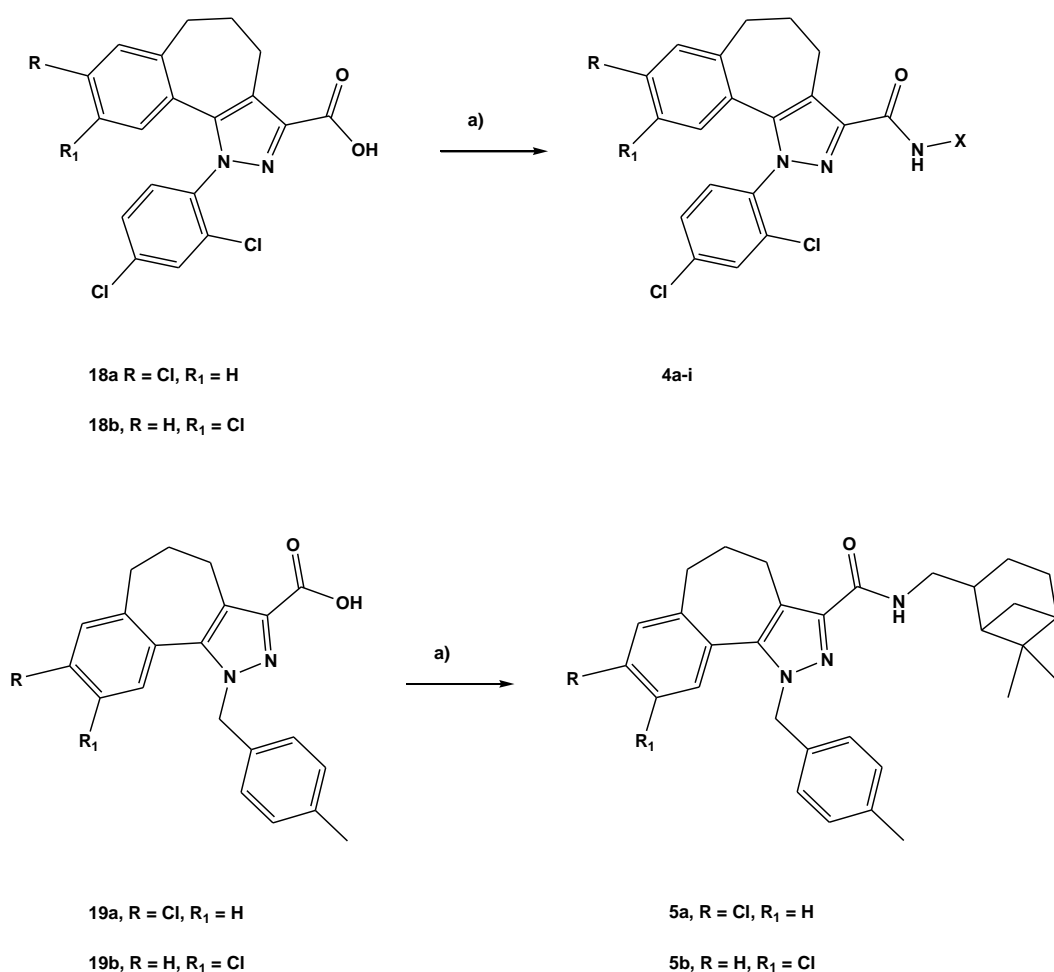
Procedura A: Sintesi di NESS0327 e analoghi carbossamidi (4a-i, 5a, e 5b)

Il 7-Clorobenzosuberone **14a** e 8-Clorobenzosuberone **14b**, sono stati sintetizzati secondo le procedure precedentemente riportate.⁸⁹ L'1,3-dichetoestere **15a-b** è stato preparato dai cloro-benzosuberoni **14a-b** per aggiunta di dietilossalato in presenza di etossido di sodio (Schema 9). Composti **15a-b** sono stati fatti reagire con 2,4-diclorofenilidrazina cloridrata o con la 4-metilbenzilidrazina cloridrata in etanolo a refluxo ottenendo così gli intermedi **16a-b** e **17a-b**.⁹⁰ Gli esteri **16** e **17** sono stati idrolizzati con KOH in metanolo per dare il corrispondente acido carbossilico **18a-b** e **19a-b** (Schema 1).



Schema 9. Reagenti e condizioni: a) Na , $(\text{COOEt})_2$, EtOH assoluto; b) EtOH ; c) KOH , MeOH .

Come mostrato nello Schema 10, gli acidi **18a-b** vengono fatti reagire con le ammine opportune o idrazine in presenza di *N*-(3-dimetilaminopropil)-*N*'-etilcarbodiimide idrocloride (EDC) e 1-idrossibenzotriazolo idrato (HOBt) per ottenere i composti desiderati **4a-i**. Allo stesso modo i carbossamidi **5a-b** sono stati ottenuti partendo da **19a-b**.

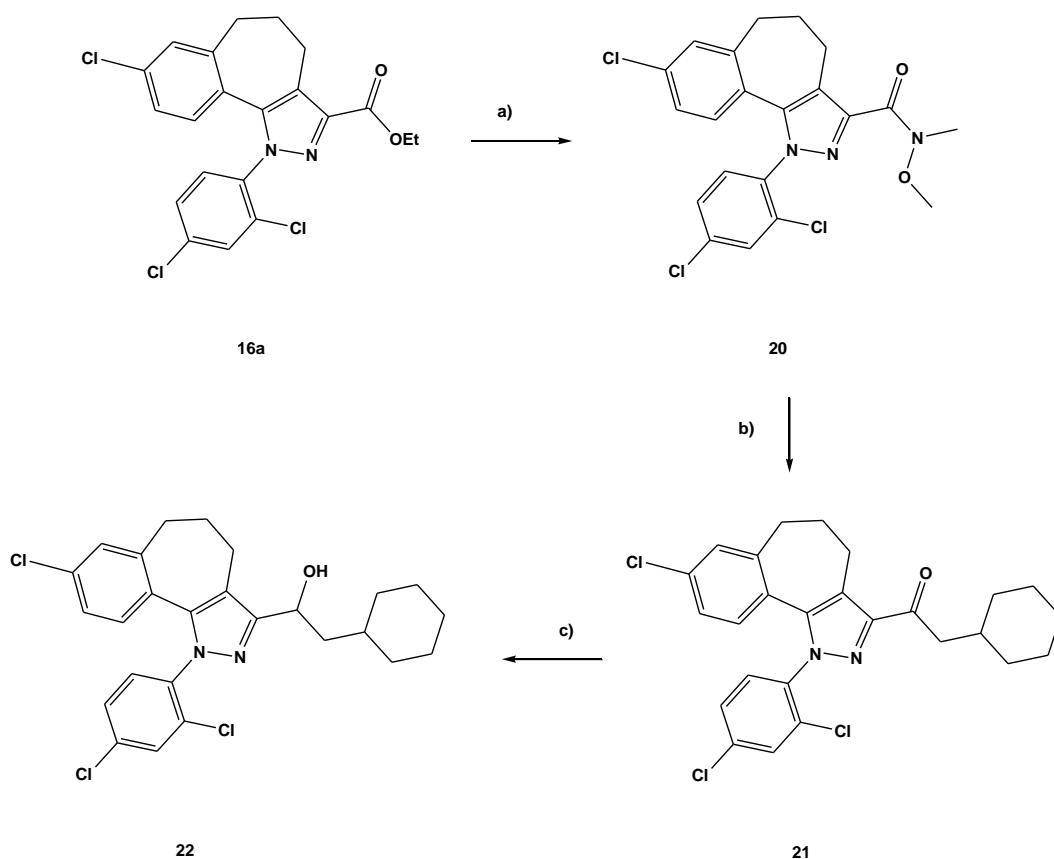


Scheme 10. Reagenti e condizioni: a) EDC, HOBt, $X-NH_2$, CH_2Cl_2 .

Sintesi dei derivati **21** e **22**

L'amide di Weinreb **21** è stata sintetizzata dall'estere **16a** dalla reazione con *N,O*-dimetildrossilamina idrocloride in presenza di trimetilalluminio (Schema 3). La reazione di **21** con (cicloesilmetil)magnesio bromuro in

tetraidrofurano anidro ha portato alla formazione del chetone triciclico **21**. Infine, il gruppo carbonilico di **21** viene ridotto da boridruro di sodio a temperatura ambiente e si ottiene la miscela racemica dell'alcol (\pm)-**22**.

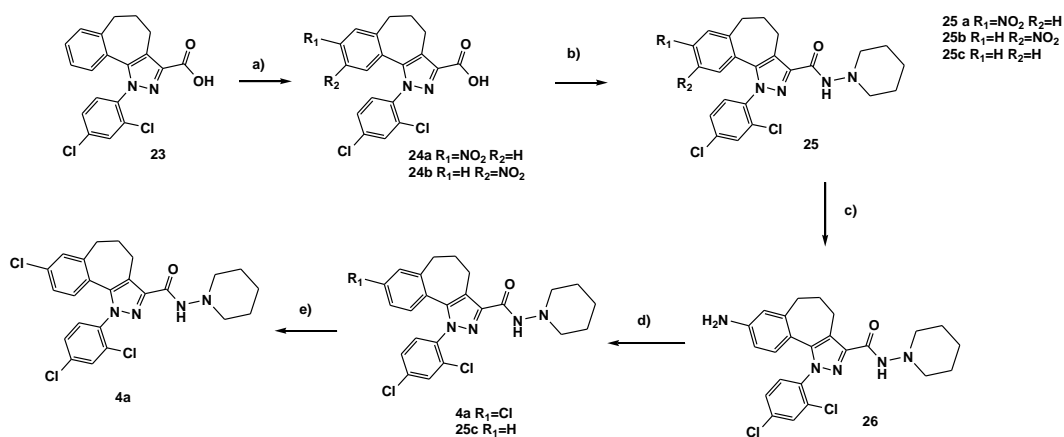


Schema 11: Reagenti e condizioni: (a) AlMe_3 , MeNHOMe HCl , CH_2Cl_2 ; (b) (cyclohexylmethyl)magnesium bromide, THF; (c) NaBH_4 , MeOH.

Procedura B: Sintesi di NESS0327

Come mostrato nello Schema 12, il secondo percorso per ottenere **4a** è stata definita secondo la procedura precedentemente riportato sviluppata da Stoit et al. (Schema 12) La nitratura del triciclico acido 1-(2',4'-dicloroenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossilico acido **23**[13], avviene per reazione di acido nitrico in acido acetico , dando una miscela di **24a** e **24b**, della resa pressoché quantitativa. Questa miscela di regioisomeri è stata fatta

reagire con 1-aminopiperidina in presenza di *O*-(Benzotriazol-1-il)-*N,N,N',N'*-tetrametiluronium esafluorofosfato (HBTU) e *N,N*-diisopropiletilamina (DIPEA), seguita dalla separazione cromatografica dei regioisomeri risultanti, per dare **25**. La riduzione del gruppo nitro in **25a**, condotta in presenza di ferro in polvere e HCl 1N, porta alla formazione dell'arilamina **26** (84% resa). Infine, il **4a** è stato ottenuto al 27% di resa da **26** mediante la reazione di Sandmeyer.



Schema 12. Reagenti e condizioni (a) HNO_3 , CH_3COOH ; (b) HBTU, DIPEA, 1-aminopiperidine, CH_2Cl_2 ; (c) Fe, 1N HCl, EtOH/ H_2O ; (d) $NaNO_2$, CuCl, 37% HCl, H_2O ; (e) chromatography on silica gel (CH_2Cl_2 /acetone 9/1).

Da questa reazione abbiamo anche ottenuto basse rese di **25c**, il corrispondente derivato non clorurato, che era difficilmente separabile mediante cromatografia in colonna. Secondo Stoit,⁸⁸ la bassa resa di **4a** e la formazione di **25c** al termine del processo sintetico era dovuta a parziale riduzione di **4a** a **25c** nella reazione Sandmeyer (stadio d di Schema 4). Tuttavia, noi crediamo invece che la piccola quantità di **25c** nel prodotto finale di questo procedimento sintetico potrebbe derivare da una nitratura incompleta dell'acido (a, Schema 12), che ha prodotto una miscela di regioisomeri nitro **24a** e **24b** contenente una piccola quantità di materiale di partenza **23**, difficile da rilevare e separare mediante cromatografia. Infatti, il **25c** era inseparabile da **25a** nelle condizioni cromatografiche utilizzate da Stoit et al. [17]. Così, **25c** è stato recuperato

immodificato insieme **4a**. Il prodotto ottenuto dalla reazione di Sandmeyer viene purificato per cromatografia su gel di silice per separare **4a** dal **25c**.

I risultati dei saggi^{77, 78} del **4a** ottenuti da entrambe le procedure di sintesi sono stati confrontati con quelli dei due composti di riferimento CP-55,940 e Rimonabant, nonché con dati K_i dei composti corrispondenti riportati in letteratura (Table 1)

Table 3 Affinità Recettori Cannabinoidi del **9a**, CP-55,940, e del Rimonabant.

Composto	K_iCB_1 (nM) ^a	K_iCB_2 (nM) ^b
4a da Procedura A	4.20 (0.3-56) ^c	55.7 (18-176) ^c
4a da Procedure B	8.18 (0.9-60) ^c	85.2 (35-208) ^c
4a ⁸⁶	0.00035±0.000005 ^d	21±0.5 ^d
4a ⁸⁸	126	not reported
4a ⁸⁷	18.4±1.62 ^d	758
CP-55,940	0.62 (0.2-1.9) ^c	9.73 (1.1-18.0) ^c
Rimonabant	2.82 (0.9-9.8) ^c	1400 (750-2350) ^c
Rimonabant [12]	1.80±0.075 ^d	514±30 ^d

^c 95% limiti di confidenza

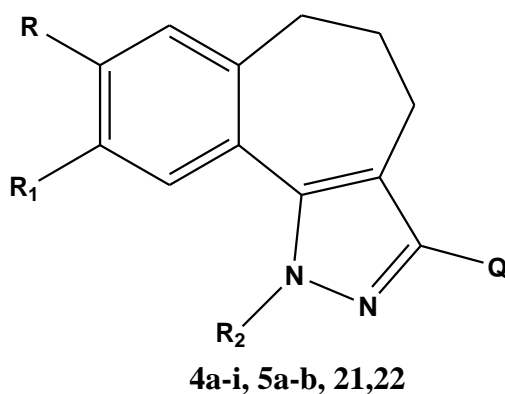
^d letteratura

Il valore affinità CB1 del campione **4a** ottenuto tramite procedura A ($K_iCB_1 = 4.20$ nM) non è d'accordo con quella precedentemente riportata da parte del nostro gruppo, ed è più in accordo con i dati riportati da Zhang.⁸⁷ Il nuovo valore nanomolare K_iCB_1 per **4a** è stata confermato anche per il composto ottenuto da Procedura B anche se c'è una piccola differenza. Tuttavia, è importante notare che i nuovi valori di K_iCB_1 di **4a** sono un ordine di grandezza inferiore a quello precedentemente misurato da Stoit (K_iCB_1 126 nM).

L'affinità verso i CB1 e CB2 è stata confrontata anche per i derivati **4b-i**, così come per i composti **5a-b**, e **21-22** (Tabella 4). Anche i derivati **4b-d**, evidenziano valori K_iCB superiori rispetto a dati precedentemente riportati, che erano dell'ordine di 0.001-0.3 nM.⁸⁹ L'introduzione di un metilene tra l'anello cicloesile e il gruppo carbossamide nella posizione 3 dell'anello pirazolico (dal **4d**

al **4e**) ha determinato un raddoppio delle K_iCB_1 , anche se questa modifica ha mantenuto buona affinità CB_1 (**4e**: $K_iCB_1=19.4$ nM). Nessun effetto significativo è stato registrato per la sostituzione dell'anello cicloesile in **4e** nei derivati **4f - h**. Tuttavia, il composto che porta il sostituito ingombrante mirtanil (**4h**) ha mostrato la migliore affinità CB_1 , con un $K_iCB_1 = 3.6$ nM, che era equivalente a quello determinato per il **4a**. Lo spostamento del atomo di cloro 8 e 9 ha indotto una diminuzione di affinità per i CB_1 , come evidenziato dal confronto dei composti **4h** e **4i**. Tuttavia, anche il derivato **4i** evidenzia una buona affinità CB_1 (**4i**: $K_iCB_1 = 17.3$ nM).

Table 4. Struttura e affinità dei composti sintetizzati.



Composto	R	R ₁	R ₂	Q	$K_iCB_1^a$	K_iCB_2	K_iCB_2/K_iCB_1
4a	Cl	H			4.2 (0.3-56)	55.7 (18-176)	13.26
4b	Cl	H			17.2 (1-244)	182 (95-348)	10.58
4c	Cl	H			4.5 (1-24)	28.1 (11-74)	6.24
4d	Cl	H			10.2 (1-97)	17.9 (10-31)	1.75
4e	Cl	H			19.4 (7-58)	31.8 (15-68)	1.64

4f	Cl	H		19.1 (7-49)	13.3 (6-28)	0.69
4g	Cl	H		24.8 (3-226)	52.1 (21-131)	2.10
4h	Cl	H		3.6 (2-7)	19.5 (13-30)	5.42
4i	H	Cl		17.3 (3-121)	13.6 (4-48)	0.79
5a	Cl	H		313 (28-3536)	9.5 (7-14)	0.03
5b	H	Cl		169 (48-587)	51.1 (18-95)	0.30
21	Cl	H		8.5 (6-13)	325 (147-722)	38.24
22	Cl	H		154 (97-245)	24 (7-88)	0.16

^a nM (95% limite di confidenza)

La sostituzione di 2,4- dicloro-fenil dei composti **4h** e **4i** con 4-metil - benzil nei composti **5a** e **5b** ha provocato una significativa riduzione di affinità CB1. Nessun effetto significativo sulla affinità CB1 è stato invece osservato con l'introduzione di un gruppo metilenico in posizione 3 dell'anello pirazolico. Il composto **21** ha infatti KiCB1 = 8,5 nM , che è equivalente a quella dell' analogo vicini **4d** . E ' importante notare che una riduzione del gruppo chetonico di **21** per ottenere **22**, ha determinato un valore KiCB1 18 volte maggiore, evidenziando una maggiore disponibilità degli elettroni dell' ossigeno del gruppo carbonilico per formare legami idrogeno con CB1 piuttosto che quelli della porzione idrossilica. Tra i composti sintetizzati, il composto **21** ha evidenziato la migliore selettività CB1, con KiCB2/KiCB1 = 38.24 (Tabella 4). Accettabile selettività

CB1 è stato registrato solo per **4a** e **4b** (K_{iCB2}/K_{iCB1} di 13.26 e 10.58 rispettivamente). Il nuovo valore determinato per il **4a** è in accordo con quanto riportato da Zhang.

Conclusioni

Abbiamo ex-novo risintetizzato NESS0327(**4a**), seguendo due percorsi alternativi in base alle procedure di sintesi precedentemente riportate. Il confronto dei dati fisico-chimici e spettrali di campioni inequivocabilmente dimostra l'equivalenza strutturale. Tuttavia, la prima procedura offriva il prodotto finale **4a** in maggiore resa e purezza di quella della seconda.

I valori di affinità CB1 dei nuovi campioni di **4a** sono in disaccordo con quelli precedentemente riportati da parte del nostro gruppo. In accordo con i dati riportati da Zhang, **4a** evidenzia valori K_{iCB1} dell'ordine di unità nM, con $K_{iCB2}/K_{iCB1}=13.26$. I nuovi dati riportati indicano che l'affinità di **4a** per i CB1 è comparabile con quella del Rimonabant. L'importanza del composto **4a** e analoghi rimane elevata considerando sia il nuovo valore di affinità sui CB1 e la conferma di profilo antagonismo del composto ri-sintetizzato per questo lavoro.

4 Conclusioni

Sono state progettate e sintetizzate tre serie di composti a struttura pirazolica e a struttura pirazolica costretta. Le linee sintetiche utilizzate sono articolate ma hanno portato all'ottenimento di composti con attività nell'ordine nM. Una volta conclusi i test biologici verrà valutata la possibilità di utilizzare alcuni di questi derivati come radioligandi, previa funzionalizzazione con ^{18}F o ^{11}C , al fine di condurre degli studi specifici sul sistema cannabinoide e studiare in modo più approfondito il suo coinvolgimento in diverse patologie.

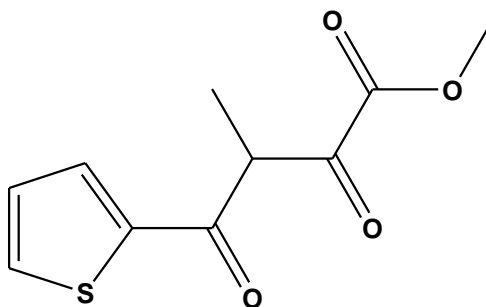
5 Materiali e metodi

Tutte le reazioni che impiegano prodotti sensibili all'aria o all'umidità sono state condotte in atmosfera di azoto ad elevata purezza. I reagenti e i solventi utilizzati per tutti gli altri scopi sono stati acquistati dalla Sigma-Aldrich Chemical Company ed utilizzati tal quali.

I punti di fusione sono stati misurati su un Apparato per il Punto di Fusione Stuart SMP10 e sono riportate senza correzione. Per la cromatografia su strato sottile sono state utilizzate lastre di gel di silice Fluka su lamina di PET o vetro con indicatore fluorescente a 254nm. La purificazione dei composti è stata ottenuta con cromatografia flash (FC) su gel di silice Merck® 60 (230-400 mesh ASTM), eluendo con miscele appropriate di solventi, eventualmente seguita da cristallizzazione da opportuni solventi. Gli spettri IR sono stati eseguiti su uno Spettrofotometro Bruker modello Alpha-T FT-IR, equipaggiato con un Alpha Eco ATR come modulo di campionamento, e sono espressi in ν (cm^{-1}). Tutti gli spettri NMR sono stati eseguiti con uno Spettrometro NMR Bruker Ultra Shield 400 Avance III, con i nuclei ^1H e ^{13}C osservati rispettivamente a 400 e a 100 MHz. I chemical shifts per il protone ed il ^{13}C sono espressi in δ rispetto al segnale del TMS ($(\text{CH}_3)_4\text{Si}$). Le molteplicità sono riportate come s (singoletto), bs (singoletto allargato), d (doppietto), t (tripletto), q (quartetto), dd (doppietto di doppietti), m (multipletto). Tutte le reazioni che coinvolgono composti sensibili all'aria o all'umidità sono state eseguite in atmosfera di azoto ad elevata purezza. Tutti i materiali, i solventi, i reagenti e i precursori e le ammine necessarie per la preparazione dei composti finali sono stati acquistati da fornitori specializzati

Procedure sintetiche dettagliati intermedi e prodotti finali della prima serie

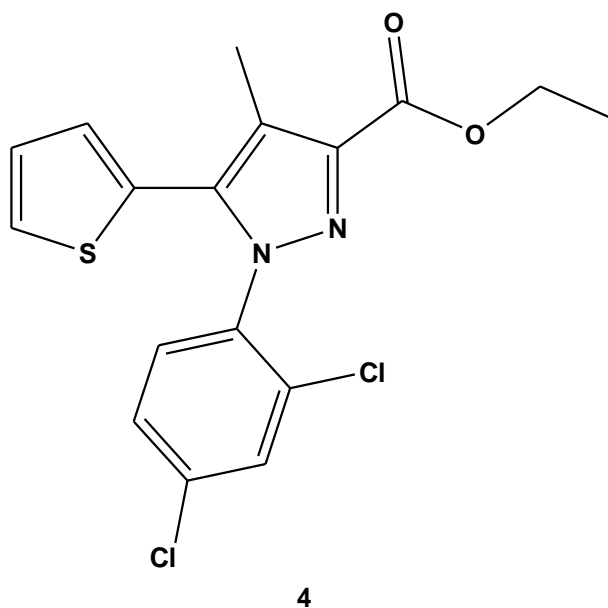
Etil-3-metil-2,4-dioxo-4-(tiofen-2-il)butanoato (3)



3

Sotto atmosfera di azoto, il sodio metallico (0,86 g, 37,50 mmoli) è stato aggiunto in piccole porzioni in etanolo (25 mL) e agitato a temperatura ambiente fino a quando tutto il sodio era dissolto. Il dietil ossalato (7,6 mL, 56,30 mmol) è stato poi aggiunto, goccia a goccia seguita da aggiunta di una soluzione di 1-(tiofen-2-il) propan-1-one (2,63g, 18,65 mmoli) in etanolo anidro (26 mL). La miscela è stata agitata a temperatura ambiente per 18 h, dopodichè viene versata lentamente in una miscela di ghiaccio e HCl 1N. La miscela risultante è stata estratta con etere e la soluzione viene concentrata. Il prodotto è stato isolato mediante cromatografia flash (etere di petrolio/acetato di etile, 8: 2) per ottenere 3,80g (85% resa) di 4 come solido bianco. $R_f = 0,57$ (etere di petrolio/acetato di etile, 8: 2); ^1H NMR (400 MHz, CDCl_3) δ : 1.24 (t, 3H, $J = 7.1$ Hz), 1.45 (d, 3H, $J = 7.0$ Hz), 4.25 (q, 2H, $J = 7.1$ Hz), 4.70 (q, 1H, $J = 7.0$ Hz), 7.37 (t, 1H, $J = 8.4$ Hz), 7.37 (d, 1H, $J = 8.4$ Hz), 7.51 (d, 1H, $J = 8.4$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 13.4, 14.0, 52.6, 63.1, 128.6, 133.5, 135.3, 142.4, 160.4, 187.5, 190.1; MS (ESI), calcolato m/z $\text{C}_{11}\text{H}_{12}\text{O}_4\text{S}$: 241.0 $[\text{M}+\text{H}]^+$, 263.0 $[\text{M}+\text{Na}]^+$, trovato m/z (relativa intensità): 241.0 $[\text{M}+\text{H}]^+$ (100), 263.0 $[\text{M}+\text{Na}]^+$

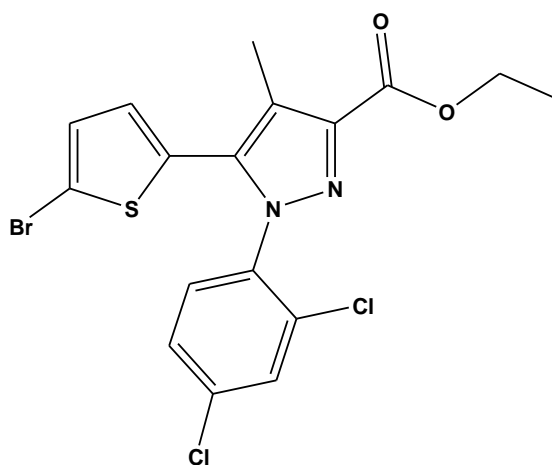
Etil 5-(tiofen-2-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazolo-3-carbossilato (4)



α – γ dichetoestere **3** è stato sciolto in EtOH assoluto (36 mL) e 2,4-diclorofenilidrazina cloridrato

(4,38 g, 20,51 mmoli) è stato aggiunta, la miscela è stata lasciata agire a riflusso per una notte. Il solvente è stato rimosso sotto pressione e il prodotto grezzo è stato purificato con la flash cromatografia (Esano/EtOAc 8: 2). A ricristallizzazione finale (Esano/EtOAc 7: 3) ha dato composto **4** (2,01 g, 32%) come solido bianco. R_f 0,30 (esano/EtOAc 8: 2);. R_f = 0.30 (petroleum ether/ethyl acetate, 8:2); ^1H NMR (400 MHz, CDCl_3) δ : 1.45 (t, 3H, J = 7.1 Hz), 2.46 (s, 3H), 4.47 (q, 2H, J = 7.1 Hz), 6.92 (dd, 1H, J = 1.2, 3.6 Hz), 7.02 (dd, 1H, J = 3.6, 5.1 Hz), 7.33 (dd, 1H, J = 2.2, 8.5 Hz), 7.38 (dd, 1H, J = 1.2, 5.1 Hz), 7.40 (d, 1H, J = 8.5 Hz), 7.46 (d, 1H, J = 2.2 Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 9.9, 14.5, 61.0, 120.0, 127.2, 127.7, 127.8, 128.6, 128.9, 130.0, 131.0, 133.9, 136.0, 136.3, 137.8, 142.9, 162.7; MS (ESI), calcolato m/z $\text{C}_{17}\text{H}_{14}\text{Cl}_2\text{N}_2\text{O}_2\text{S}$: 381.0 $[\text{M}+\text{H}]^+$, 383.0 $[\text{M}+2+\text{H}]^+$, trovato m/z (intensità relativa): 381.0 $[\text{M}+\text{H}]^+$ (100), 383.0 $[\text{M}+2+\text{H}]^+$

Etil 5-(5-bromotiofen-2-il)-1-(2,4-dichlorofenil)-4-metil-1H-pirazolo-3-carbossilato (5)

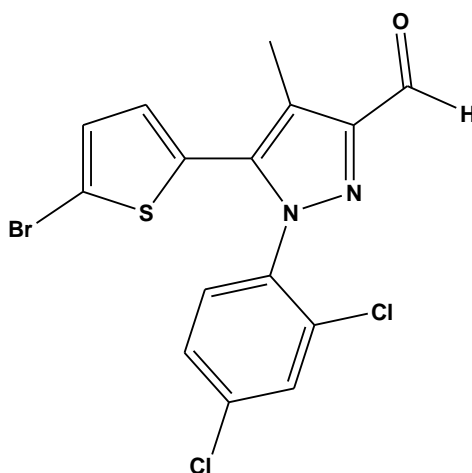


5

2.21 g di **4** (1.31 mmol) vengono sciolto in acetonitrile (4,5 mL) e la soluzione è stata raffreddata a 0 °C. NBS (0,39 g, 2,23 mmol) è stato aggiunto in piccole porzioni, e la miscela viene lasciata agire per una notte a RT. Una soluzione satura di Na₂S₂O₃ (5 mL) è stata aggiunta e il solvente è stato rimosso sotto pressione ridotta. La miscela risultante è stata estratta con EtOAc, gli strati organici sono stati lavati con acqua, salamoia, anidrificata su MgSO₄, filtrata e il solvente è stato evaporato a pressione ridotta. Il prodotto viene purificato mediante flash cromatografia (Esano/EtOAc 8: 2) a dare il composto **5** (510 mg, 83%) come solido giallo pallido. *R_f* = 0.38 (Esano/AcOEt, 8:2) *R_f* 0.38 (Esano/EtOAc 8:2); ¹H NMR (400 MHz, CDCl₃) δ: 1.40 (t, 3H, *J* = 7.1 Hz), 2.41 (s, 3H), 4.43 (q, 2H, *J* = 7.1 Hz), 6.63 (d, 1H, *J* = 3.9 Hz), 6.94 (d, 1H, *J* = 3.9 Hz), 7.33 (d, 1H, *J* = 2.0 Hz), 7.35 (d, 1H, *J* = 0.4 Hz), 7.45 (dd, 1H, *J* = 0.4, 2.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ: 10.1, 14.6, 61.2, 115.1, 120.5, 128.0, 129.4, 130.3, 130.3, 131.0, 133.9, 134.1, 135.8, 136.7, 137.0, 142.8, 162.6; MS (ESI),

calcolato m/z C₁₇H₁₃ 78Br₃₅Cl₂N₂O₂S: 458.9 [M+H]⁺, 460.9[M+2+H]⁺,
trovato m/z (intensità relativa): 458.9 [M+H]⁺ (65) 460.9 [M+2+H]⁺ (100).

**5-(5-bromotiofen-2-il)-3-(2,4-diclorofenil)-5-metilciclopenta-1,4-
dienecarbaldeide (6)**

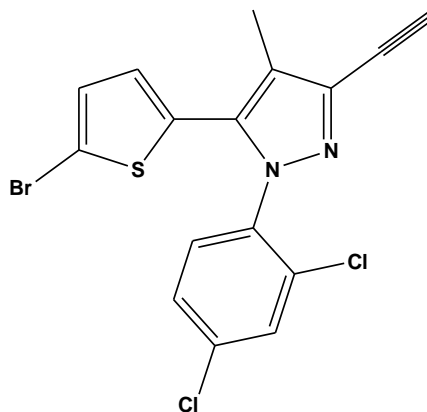


6

L' estere 5 (0,2 g, 0,43 mmoli) è stato sciolto in diclorometano anidro (2 mL) e la miscela è stata raffreddata a 78 °C. DIBAL-H (0,5 mL) è stato aggiunto goccia a goccia, la miscela viene lasciata agire per 4 ore a 78 °C. Infine è stato aggiunto MeOH (0,5 mL) e il solvente è stato rimosso sotto pressione ridotta. La miscela risultante viene estratta con EtOAc, gli strati organici sono stati lavati con salamoia, anidrificata su MgSO₄, filtrata e il solvente è stato evaporato a pressione ridotta. Il prodotto grezzo viene purificato con la flash cromatografia (Esano/EtOAc 5: 5) per dare aldeide 6 Resa= 55%. Solido bianco. R_f 0,50 (esano/EtOAc 5: 5); NMR (400 MHz, CDCl₃) δ: 2.36 (s, 3H), 6.58 (d, 1H, J = 3.9 Hz), 6.90 (d, 1H, J = 3.9 Hz), 7.29 (d, 1H, J = 0.5 Hz), 7.30 (d, 1H, J = 2.0 Hz), 7.46 (dd, 1H, J = 0.5, 2.0 Hz), 10.0 (s, 1H); MS (ESI), calcolato m/z C₁₅H₉78Br₃₅Cl₂N₂O₂S: 414.9 [M+H]⁺, 416.9 [M+2+H]⁺, trovato m/z (intensità relativa): 414.9 [M+H]⁺ (55), 416.9 [M+2+H]⁺ (100).

2-Bromo-5-(5-(2,4-diclorofenil)-3-etinil-2-metilciclopenta-1,3-dienil) tiofene

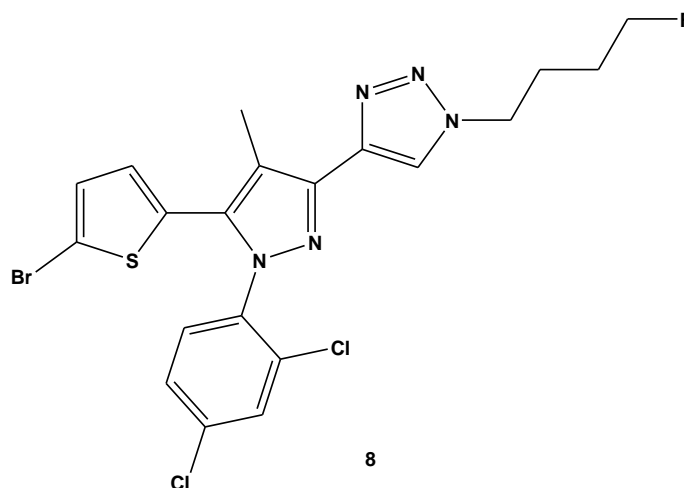
(7)



7

K_2CO_3 (0,11 g, 0,86 mmoli) e dimetil 1-diazo-2-ossopropilfosfonato (0,09 g, 0,52 mmoli) sono stati aggiunti a una soluzione dell'aldeide **6** (0,45 g, 1,09 mmol) in MeOH (0,5 mL). Dopo 5 min il bagno di ghiaccio è stato rimosso e la reazione è stata lasciata a RT per 12 h. Infine sono stati aggiunti il Sale di Rochelle (2 mL) e Et_2O (2 mL). La fase organica viene lavata con salamoia, anidrificata su MgSO_4 , filtrata ed il solvente è stato evaporato a pressione ridotta. Il prodotto grezzo è stato purificato con la flash cromatografia (Esano/EtOAc 7:3) ottenendo così l'alchino **7**. Resa= 55% .Solido bianco. Rf 0.82 (Esano/EtOAc 7:3); ^1H NMR (400 MHz, CDCl_3) δ : 2.28 (s, 3H), 3.31 (s, 1H), 6.62 (d, 1H, J = 3.9 Hz), 6.96 (d, 1H, J = 3.9 Hz), 7.36 (d, 2H, J = 1.3 Hz), 7.50 (t, 1H, J = 1.3 Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 9.4, 75.0, 81.4, 114.5, 120.1, 127.9, 128.4, 130.1, 130.2, 130.7, 130.9, 133.7, 135.2, 135.8, 136.3, 136.4; MS(ESI), calcolato m/z $\text{C}_{16}\text{H}_9\text{BrCl}_2\text{N}_2\text{S}$: 410.9 $[\text{M}+\text{H}]^+$, 412.9 $[\text{M}+2+\text{H}]^+$, 432.9 $[\text{M}+\text{Na}]^+$, 434.9 $[\text{M}+2+\text{Na}]^+$, trovato m/z (relative intensità): 410.9 $[\text{M}+\text{H}]^+$ (35), 412.9 $[\text{M}+2+\text{H}]^+$ (70), 432.9 $[\text{M}+\text{Na}]^+$ (55), 434.9 $[\text{M}+2+\text{Na}]^+$ (100).

4-(5-(5-bromotiofen-2-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-il)-1-(4-fluorobenzil)-1H-1,2,3-triazolo (8)



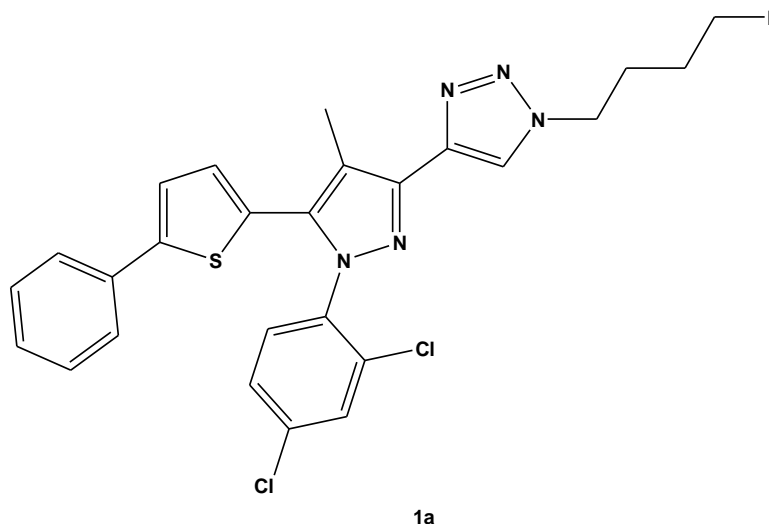
L'ascorbato di sodio (0,08 g , 0,38 mmol) e solfato di rame(0,02 g , 0,07 mmoli) sono stati aggiunti a una soluzione di alchino **7** (0,80 g , 1,94 mmol) e 1 - azido - 4 - fluorobutano (0,34 g , 2,11 mmoli) in butanolo/acqua (50 mL , 4 : 1). La miscela è stata agitata a r.t. per 24 h. Una soluzione acquosa saturata di cloruro di ammonio (20 ml) è stata aggiunta e la fase acquosa è stata estratta con EtOAc. Il solvente è stato evaporato a pressione ridotta, purificato mediante cromatografia flash (esano/EtOAc 6:4) si ottiene il composto **8** –Resa= 55%) come solido bianco. R_f 0,24 (esano /EtOAc 6: 4); ¹H NMR (400 MHz, CDCl₃) δ: 1,60-1,68 (m, 1H), 1,69-1,76 (m, 1H), 1,99-2,09 (m, 2H), 2,47 (s, 3H), 4,41 (dt, 2H, J_{H-F} = 47,2 Hz, J_{H-H} = 5,7 Hz), 4,41 (t, 2H, J = 7,0 Hz), 6,60 (d, 1H, J = 3,9 Hz), 6,89 (d, 1H, J = 3,9 Hz), 7,25-7,32 (m, 2H), 7,42 (d, 1H, J = 2,1 Hz), 7,88 (s, 1H); ¹⁹F NMR (376,45 MHz, CDCl₃) δ: 219,3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: 10,3, 26,7 (d, J_{C-F} = 4,1 Hz), 26,7 (d, J_{C-F} = 20,1 Hz), 50,0, 83,3 (d, J_{C-F} = 165,5 Hz), 114,5, 121,3, 128,1, 128,9, 130,3, 130,4, 131,2, 134,1, 136,2, 136,3, 140,2, 142,4, 144,1, 147,0, 150,5; MS (ESI), m calcolato/z C₂₀H₁₇BrCl₂FN₅S: 528,0 [M+H]⁺, 530,0 [M+2+H]⁺, 550,0 [M+Na]⁺, 552,0 [M+2+Na]⁺, trovato m/z (intensità relativa): 528,0 [M+H]⁺ (30), 530,0 [M+2+H]⁺ (65), 550,0

$[M+Na]^+$ (52), 552.0 $[M+2+Na]^+$ (100); HRMS Calc. per $C_{20}H_{18}BrCl_2FN_5S$: 527,9822 e 529.9799, trovato: 527.9813 e 529,9787

Procedura Generale I per la preparazione dei composti 1a-c

Una miscela di alchino **8** (0,18 mmol), $Pd(PPh_3)_4$ (0,09 mmol), l' appropriato acido boronico (0,28 mmol) e Na_2CO_3 0,23 mmol) in DME (2 mL), è stata riscaldata a riflusso e agitata durante la notte. La reazione è stata raffreddata a temperatura ambiente (RT), versata in acqua, ed estratta con diclorometano, anidrificata su $MgSO_4$, filtrata e il solvente è stato evaporato a pressione ridotta. Il prodotto grezzo è stato purificato con la flash cromatografia (Esano/EtOAc 6: 4) per dare il composto desiderato **1a-c**.

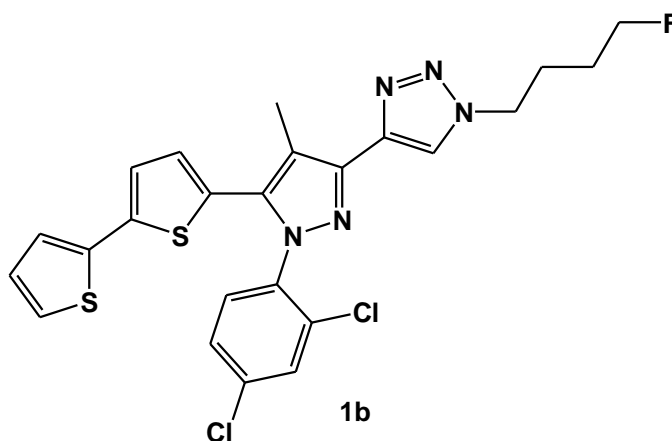
4-(5-(2,2'-bitiofen-5-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-il)-1-(4-fluorobutil)-1H-1,2,3-triazolo



Olio giallo Resa=35%.. R_f 0,36 (esano/EtOAc 6: 4); 1H NMR (400 MHz, $CDCl_3$) δ : 1,67-1,90 (m, 2H), 2,07-2,22 (m, 2H), 2,63 (s, 3H), 4,52 (dt, 2H, J_{H-F} = 47,2 Hz, J_{H-H} = 5.7 Hz), 4,52 (t, 2H, J = 6.9 Hz), 6.88 (d, 1H, J = 3.8 Hz), 7.22 (d, 1H, J = 3,8 Hz), 7,30-7,46 (m, 6H), 7.51 (d, 1H, J = 2.2 Hz), 7.56 (d, 1H, J = 2.2 Hz), 7.98 (s, 1H); ^{19}F NMR (376,45 MHz, $CDCl_3$) δ : 219,3 (s, 1F); ^{13}C NMR

(100 MHz, CDCl₃) δ : 10.3, 26.5 (d, JC-F = 4.1 Hz), 27.3 (d, JC-F = 20.1 Hz), 49.8, 83.2 (d, JC-F = 165.7 Hz), 121.2, 123.1, 125.8, 127.9, 128.0, 128.3, 129.0, 129.2, 129.7, 130.2, 130.4, 131.1, 133.6, 134.0, 135.9, 136.5, 136.8, 137.3, 142.5, 143.9, 146.2; MS (ESI), m/z calcolato C₂₆H₂₂O₃Cl₂FN₅S: 526,1 [M + H]⁺, 528,1 [M + 2 + H]⁺, trovato m/z(intensità relativa): 526,1 [M + H]⁺ (100), 528,1 [M+2+H]⁺ (70); HRMS Calc. per C₂₆H₂₃Cl₂FN₅S: 526,1030, trovata: 526.1026

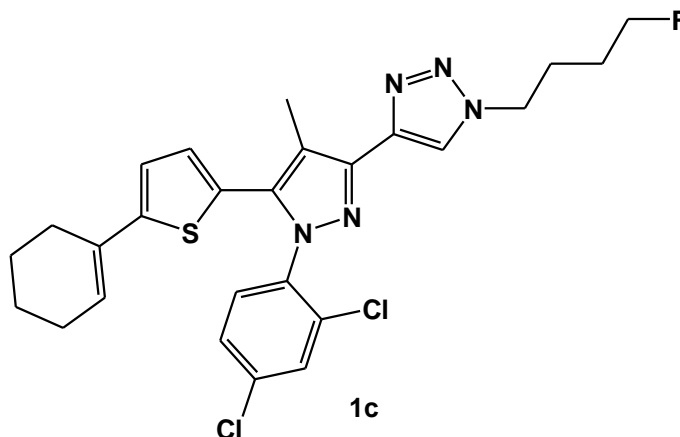
4-(1-(2,4-diclorofenil)-4-metil-5-(5-phenylthiophen-2-yl)-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1b)



Olio giallo. Resa= 35%. R_f 0,31 (esano/EtOAc 6: 4); ¹H NMR (400 MHz, CDCl₃) δ : 1,71-1,79 (m, 1H), 1,79-1,86 (m, 1H), 2,10-2,20 (m, 2H), 2.62 (s, 3H), 4,52 (dt, 2H, J_{H-F} = 47,2 Hz, J_{HH} = 5.7 Hz), 4.52 (t, 2H, J = 7.0 Hz), 6.81 (d, 1H, J = 3.8 Hz), 7.04 (dd, 1H, J = 3.6, 5.1 Hz), 7.08 (d, 1H, J = 3.8 Hz), 7.17 (dd, 1H J = 1.1, 3.6 Hz), 7.26 (dd, 1H, J = 1.1, 5.1 Hz), 7.38 (dd, 1H, J = 2.2, 8.4), 7.45 (d, 1H, J = 8.4 Hz), 7.52 (d, 1H, J = 2.2 Hz), 8.00 (s, 1H); ¹⁹F NMR (376,45 MHz, CDCl₃) δ : 219,3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ : 10.2, 26.5 (d, JC-F = 4.2 Hz), 27.3 (d, JC-F = 20.1 Hz), 49.8, 83.2 (d, JC-F = 165.7 Hz), 115.5, 120.7, 123.6, 124.2, 125.0, 127.9 (2C), 129.0, 130.2, 131.1, 134.0, 136.0, 136.3, 136.5, 137.1, 139.3, 142.4, 143.9, 146.1; MS (ESI), m calcolato/z C₂₄H₂₀Cl₂FN₅S₂: 532,1 [M+H]⁺, 534,1 [M+2+H]⁺, trovato m/z (intensità

relativa): 532,1 $[M+H]^+(100)$, 534,1 $[M+2+H]^+(75)$; HRMS Calc. per $C_{24}H_{21}Cl_2FN_5S_2$:532.0594, trovata: 532,0588.

4-(5-(5-cicloeseniltiofen-2-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-il)-1-(4-fluorobutil)-1H-1,2,3-triazolo (1c)



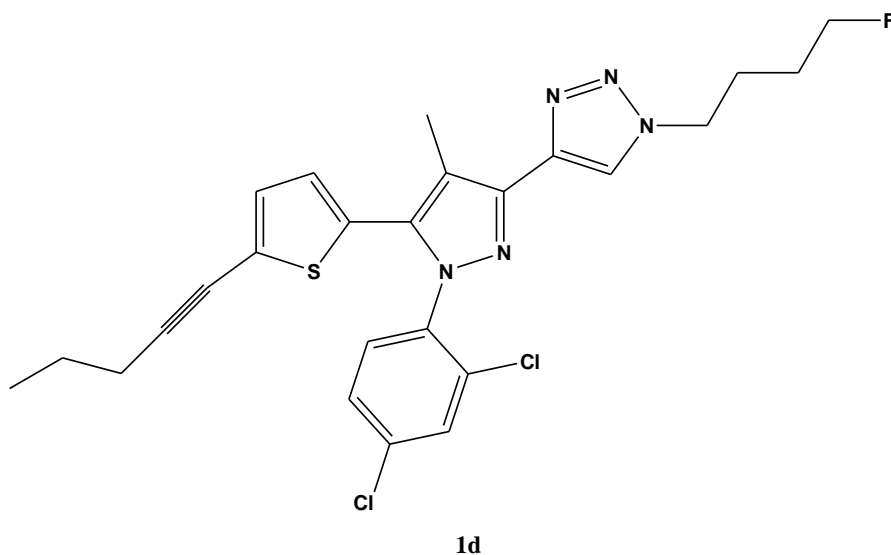
Olio giallo . Resa= 75%. Rf 0,36 (esano/EtOAc6: 4); 1H NMR (400 MHz, $CDCl_3$) δ : 1,58-1,71 (m, 4H), 1,71-1,84 (m, 4H), 2,16-2,23 (m, 2H), 2,34-2,40 (m, 2H), 2,58 (s, 3H), 4,51 (t, 2H, $J = 7,0$ Hz), 4,51 (DT, 2H, $J_{H-F} = 47,2$ Hz, $J_{H-H} = 5,7$ Hz), 6,15-6,18 (m, 1H), 6,73 (d, 1H, $J = 3,8$ Hz), 6,82 (d, 1H, $J = 3,8$ Hz), 7,34 (dd, 1H, $J = 2,2, 8,5$ Hz), 7,40 (d, 1H, $J = 8,5$ Hz), 7,50 (d, 1H, $J = 2,2$ Hz), 7,98 (s, 1H); ^{19}F NMR (376,45 MHz, $CDCl_3$) δ : 219,3 (s, 1F); ^{13}C NMR (100 MHz, $CDCl_3$) δ : 10,2, 22,0, 22,6, 25,6, 26,5 (d, $J_{C-F} = 4,0$ Hz), 27,4 (d, $J_{C-F} = 22,0$ Hz), 29,7, 49,8, 83,2 (d, $J_{C-F} = 165,7$ Hz), 115,2, 121,1, 125,2, 126,3, 127,8, 128,1, 128,5, 130,1, 130,3, 130,7, 131,1, 134,0, 135,7, 136,6, 142,0, 143,8, 149,0; MS (ESI), calcolato m/z $C_{26}H_{26}FCl_2N_5S$: 530,1 $[M+H]^+$, 532,1 $[M+2+H]^+$, trovato m/z (intensità relativa): 530,1 $[M+H]^+$ (100), 532,1 $[M+2+H]^+$ (70); HRMS Calc. per $C_{26}H_{27}Cl_2FN_5S$: 530,1333, trovato: 530,1343.

Procedura Generale II per la Preparazione dei composti 1d-e.

Una miscela di alchino **8** (0,09 mmol), $Pd(PPh_3)_4$ (0,003 mmoli), DIPEA (1 mL) e l'alchino appropriato (0,19 mmol di entrambi 1-pentino o

ciclopropilacetilene) è stata agitata a 40 °C per 20 min. Infine è stato aggiunto CuI (0,006 mmol) e la reazione è stata lasciata sotto agitazione a 80 °C durante la notte. La miscela è stata raffreddata a RT., diluito con EtOAc (1 mL), anidrificata su MgSO₄, filtrata e il solvente è stato evaporato a pressione ridotta. Il prodotto grezzo è stato purificato prima mediante HPLC (semi-preparativa colonna C18 Luna, Eluente: A: H₂O, Eluente B: CH₃CN; in condizioni isocratiche Eluente A: 20% e Eluente B: 80%, 5 mL/min) per dare il composto desiderato. 1d-e

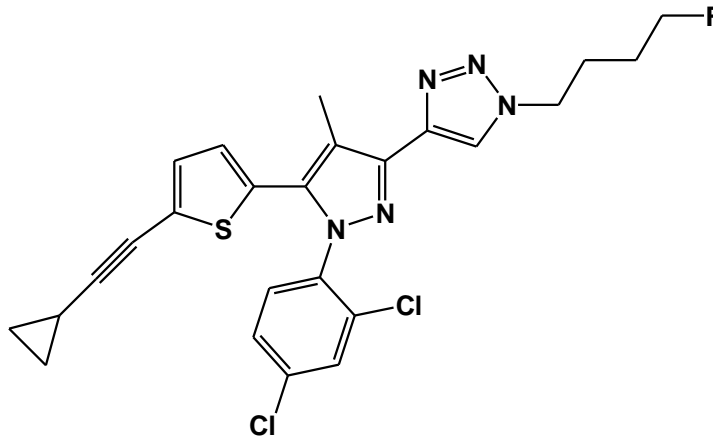
4-(1-(2,4-dichlophenyl)-4-methyl-5-(5-(pent-1-ynyl)thiophen-2-yl)-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1d)



La procedura generale II è stata usata per convertire 8 e 1-pentino nel prodotto sopra riportato. Olio giallo dopo una purificazione HPLC , con colonna semipreparativa, Eluente A: H₂O, Eluente B: CH₃CN; in condizioni isocratiche Eluente A: 20% e Eluente B: 80%, 5 mL/min, tempo di ritenzione: 8,9 min). R_f 0,24 (esano/EtOAc 6: 4); ¹H NMR (400 MHz, CDCl₃) δ: 1.02 (t, 3H, J = 7,4 Hz), 1,52-1,66 (m, 2H), 1,68-1,75 (m, 1H), 1,75-1,85 (m, 1H), 2,04-2,19 (m, 2H), 2.39 (t,

2H, J = 7.1 Hz), 2.55 (s, 3H), 4.48 (t, 2H, J = 7.0 Hz), 4.49 (dt, 2H, JHF = 47.2 Hz, JH-H = 5.7 Hz), 6.77 (d, 1H, J = 3.8 Hz), 6.99 (d, 1H, J = 3.8 Hz), 7.33 (dd, 1H, J = 2.1, 8.4 Hz), 7.38 (d, 1H, J = 8.4 Hz), 7.48 (d, 1H, J = 2.1 Hz), 7.96 (s, 1H); ¹⁹F NMR (376,45 MHz, CDCl₃) d: ? 219,3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: 10.3, 13.7, 21.8, 22.1, 26.5 (d, JC-F = 4.2 Hz), 27.3 (d, JC-F = 20,1 Hz), 29,8, 50,0, 73,3, 83,3 (d, JC-F = 165,7 Hz), 96,6, 115,6, 116,5, 121,3, 126,4, 128,1, 130,4, 131,1, 131,2, 134,1, 136,1, 136,3, 137,0, 142,4, 144,0; MS (ESI), calcolato m/z C₂₅H₂₄Cl₂FN₅S: 516,1 [M+H]⁺, 518,1 [M+2+H], trovato m/z (intensità relativa): 516,1 [M+H]⁺ (100), 518,1 [M+2+H] (75); HRMS Calc. per C₂₅H₂₅Cl₂FN₅S: 516,1186, trovato: 516,1176.

4-(5-(ciclopropil-etinil)-tiofen-2-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-il)-1-(4-fluorobutil)-1H-1,2,3-triazolo (1e)



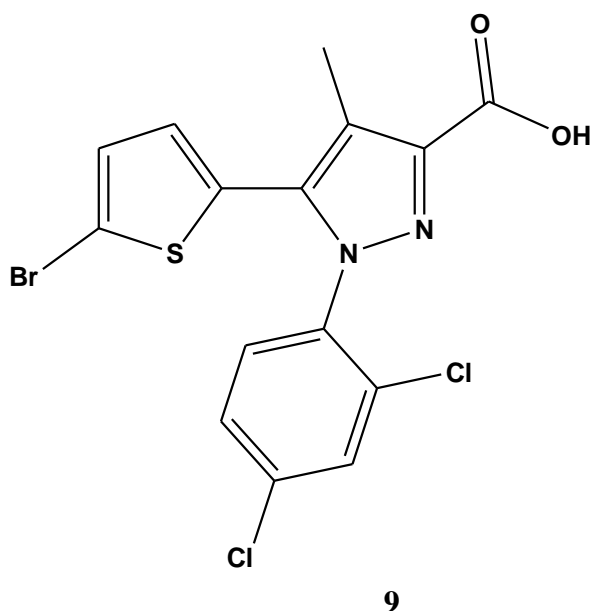
1e

La procedura generale II è stata usata per convertire 8 e il ciclopropilacetilene nel prodotto sopra riportato. Olio giallo (40 mg, 25%) dopo una purificazione con HPLC, colonna semi-preparativa, eluente: A: H₂O, eluente B: CH₃CN; in condizioni isocratica Eluente A: 20% e Eluente B: 80%, 5 mL/min, Tempo di ritenzione: 12,0 min. R_f 0,24 (esano/EtOAc 6: 4); ¹H NMR (400 MHz, CDCl₃) δ: 0,77-0,86 (m, 2H), 0,87-0,94 (m, 2H), 1,41-1,54 (m, 1H), 1,70-1,77

(m, 1H), 1,77-1,85 (m, 1H), 2,08-2,19 (m, 2H), 2.56 (s, 3H), 4.51 (t, 2H, J = 7.0 Hz), 4.51 (dt, 2H, JH-F = 47,2 Hz, JH-H = 5,7 Hz), 6,69 (d, 1H, J = 3.9 Hz), 6.98 (d, 1H, J = 3.9 Hz), 7,33-7,41 (m, 2H), 7.51 (d, 1H, J = 2.0 Hz), 7.98 (s, 1H); ¹⁹F NMR (376,45 MHz, CDCl₃) δ: 219,3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: 1,47, 8,50, 8,68 (2C), 26,5 (d, JC-F = 4,1 Hz), 27,0 (d, JC-F = 19,2 Hz), 46,1, 49,8, 68,0, 83,2 (d, JC-F = 165,5 Hz), 90,2, 115,6, 118,7, 121,1, 126,1, 126,7, 127,9, 128,5, 129,5, 130,1, 131,2, 133,9, 135,9, 137,6, 143,4; MS (ESI), m calcolato/zC₂₅H₂₂ 35Cl₂FN₅S: 514,1 [M+H]⁺, 516,1 [M+2+H], trovato m/z (intensità relativa): 514,1 [M+H]⁺ (100), 516,1 [M+2+H] (75); HRMS Calc. per C₂₅H₂₃Cl₂FN₅S: 514,1030, trovato: 514,1020.

Procedure sintetiche dettagliati intermedi e prodotti finali della seconda serie

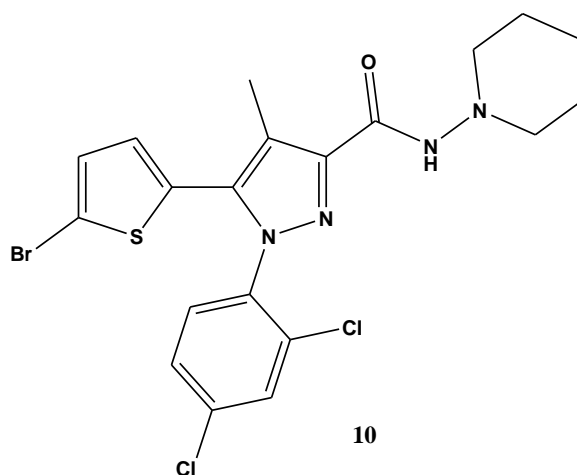
5-(5-bromotiofen-2-il)-1-(2,4-diclorofenil)-4-metil-1H-pirazol-3-carbossilico acido (9)



Ad una soluzione di etil 5- (5-bromotiofen-2-il) -1- (2,4-diclorofenil) -4-metil-1H-pirazolo-3-carbossilato **5** (1 eq., 6,3 mmoli) in metanolo (30 mL) è stato aggiunto del KOH (3 eq) .La miscela viene riscaldata a riflusso per una notte. La

soluzione è stata lasciata raffreddare a temperatura ambiente e quindi versata in una soluzione di HCl 5% (30 mL). Il precipitato ottenuto viene filtrato, lavato con acqua ed essiccato all'aria per dare l'acido analiticamente puro. L'acido analiticamente puro **6** è ottenuto come solido bianco (0.87g, resa 64,3%). $R_f = 0.50$ (CHCl₃/MeOH, 9: 1); mp: 218-220 ° C; IR: $\nu = 1690, 2617$; ¹H NMR (400MHz, DMSO) $\delta = 2.30$ (s, 3H), 6.88 (d, 1H, J = 3.9 Hz), 7.21 (d, 1H, J = 3.9 Hz), 7.60 (dd, 1H, J = 2.2, 8.4 Hz), 7.67 (d, 1H, J = 8.4 Hz), 7.85 (d, 1H, J = 2.2Hz); ¹³C NMR (100MHz, CDCl₃) $\delta = 10.3, 114.7, 119.8, 129.2, 130.1, 130.3, 131.0, 131.6, 132.4, 133.2, 136.0, 136.4, 136.9, 143.5, 164.0$; ESI-MS (MeOH): m/z Calc. 537,3 [M+Na] ⁺, trovato 537,0.

5-(5-bromotiofen-2-il)-1-(2,4-diclorofenil)-N-(piperidin-1-il)-4-metil-1H-pirazol-3-carbossamide (10)



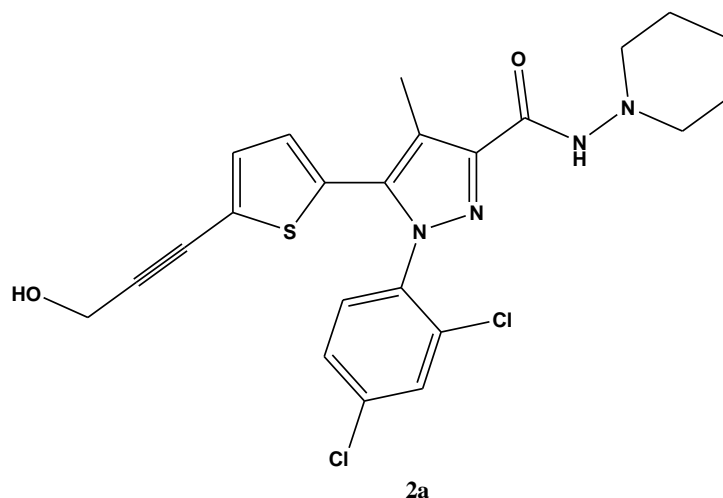
Una miscela di 5- (5-bromotiofen-2-il) -1- (2,4-diclorofenil), acido -4-metil-1H-pirazolo-3-carbossilico **9** (1 eq., 5,44 mmoli), N- (3-dimetilamminopropil) -N'-etilcarbodiimide cloridrato (1,2 eq.) e 1-idrossibenzotriazolo idrato (1.2 eq.) in CH₂Cl₂ (22 ml) è stata agitata a temperatura ambiente. Dopo mezz'ora è stata aggiunta goccia a goccia 1-aminopiperidina, la soluzione viene lasciata reagire a temperatura ambiente per 16 ore. Il solvente è stato evaporato e il prodotto analiticamente puro è stato isolato mediante cromatografia flash (etere di petrolio/acetato di etile 7: 3) seguita da ricristallizzazione (etere di petrolio/EtOAc, 6: 4) fino a ottenere **7** (0.77g, resa 78,5%) come solido bianco. $R_f = 0,34$ (etere di petrolio/EtOAc, 6: 4); mp: 142-

147°C; IR : ν = 1661, 3218; ^1H NMR (400MHz, CDCl_3) δ =1.41-1.46 (m, 2H), 1.72-1.78 (m, 4H), 2.46 (s, 3H), 2.85 (t, 4H, J =4.9 Hz); 6.64 (d, 1H, J =3.9 Hz), 6.95 (d, 1H, J =3.9 Hz), 7.33 (d, 2H, J =8.8 Hz), 7.37 (dd, 1H, J =1.9, 8.8 Hz), 7.51 (d, 1H, J =1.9 Hz), 7.59 (bs, 1H); ^{13}C NMR (100MHz, CDCl_3) δ =9.7, 23.5, 25.6, 57.2, 115.0, 119.5, 128.1, 129.2, 130.3, 130.4, 130.5, 130.9, 133.9, 135.8, 136.7, 137.1, 144.5, 159.8. ESI-MS (MeOH): m/z calcd. 537.3 $[\text{M} + \text{Na}]^+$, trovato 537.0.

Procedure Generale II: sintesi di 5-idrossialchinil Carbossamidi (2a-e).

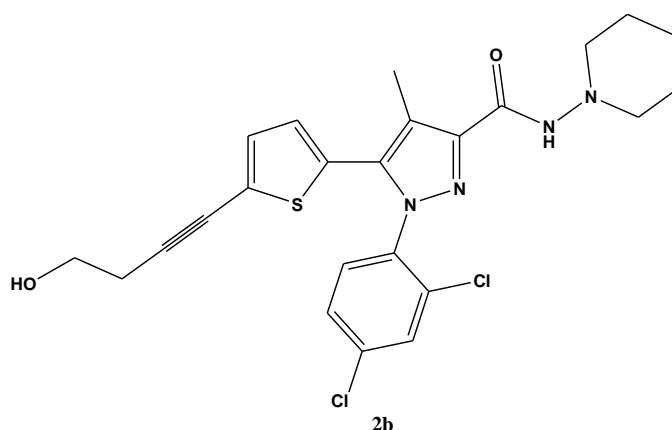
Sotto atmosfera di azoto, un pallone a fondo tondo a tre colli è stato caricato di 5-(5-bromotiofen-2-il)-1-(2,4-diclorofenil)-N-(piperidin-1-il)-4-metil-1H-pirazol-3-carbossamide 7 (1 eq., 1,55 mmoli), tetrakis (trifenilfosfina) palladio (0) (0,01 eq., 0.018g), DIPEA (5.9 ml) e l'idrossialchino appropriato (1,4 eq., 0,183 g). La miscela è stata fatta reagire a 40° C per 20 minuti, poi è stato aggiunto CuI (0,02 eq, 0.0059g). La reazione è stata lasciata reagire 12 h, poi la miscela è stata lasciata raffreddare a temperatura ambiente e diluito con AcOEt. Estratta, anidrificata con Na_2SO_4 il solvente è stato rimosso ed il prodotto analiticamente puro è stato isolato mediante cromatografia flash, come indicato di seguito.

1-(2,4-diclorofenil)-5-(5-(3-idrossiprop-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (2a).



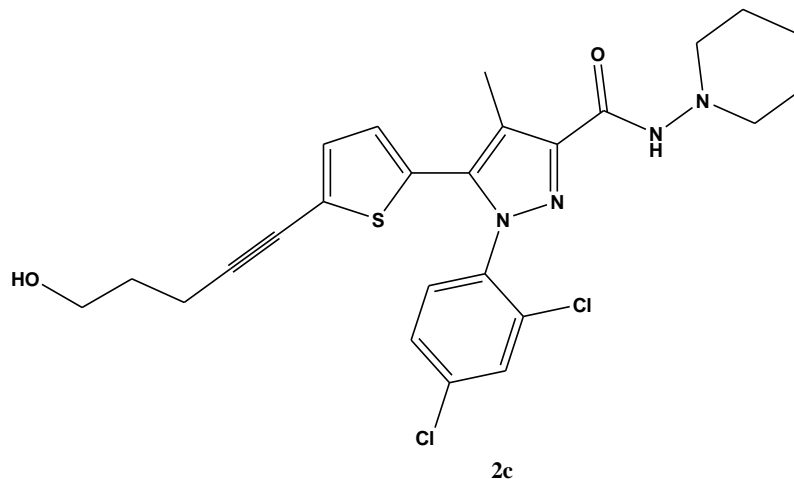
La procedura generale **II** è stata usata per convertire **10** e il propargil alcol in **2a**. Il prodotto grezzo è stato purificato mediante flash cromatografia (CH₂Cl₂/MeOH, 98:2) Solido giallo Resa 80%. R_f = 0.36 (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ=1.40-1.48 (bs, 2H), 1.71-1.82 (m, 5H), 2.47 (s, 3H), 2.80-2.88 (m, 4H), 4.48 (d, 2H, J= 6.02 Hz), 6.73 (d, 1H, J=3.8 Hz), 7.06 (d, 1H, J= 3.8 Hz), 7.32 (d, 1H, J=8.5 Hz), 7.35 (dd, 1H, J= 2.0, 8.5 Hz), 7.48(d, 1H, J= 2.0 Hz), 7.59 (bs, 1H); ESI-MS (MeOH): m/z calcd. 389.08 [M+H]⁺, trovato 389.1.

1-(2,4-diclorofenil)-5-(5-(4-idrossibut-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (2b).



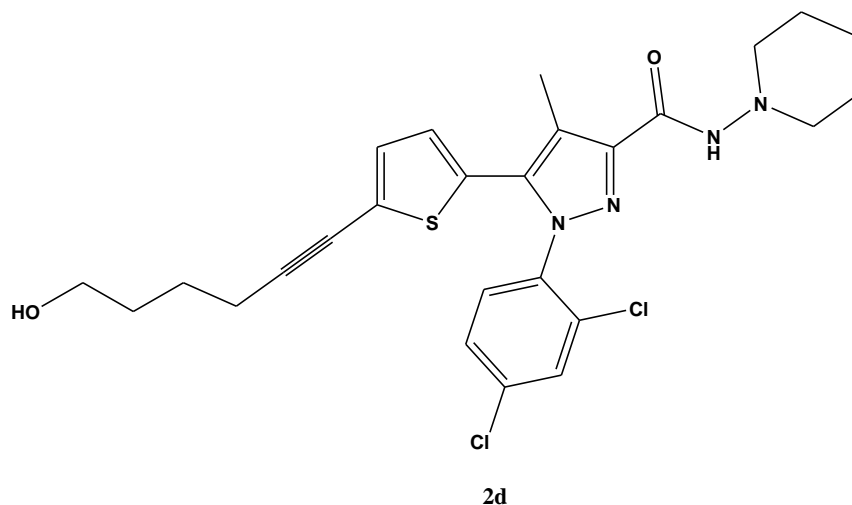
La procedura generale **II** è stata usata per convertire **10** e il 3-butin-1-olo in **2b**. Il prodotto grezzo è stato purificato mediante flash cromatografia (CH₂Cl₂/MeOH, 98:2) Resa: 93% Solido giallo R_f = 0.32 (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ=1.40-1.48 (bs, 2H), 1.71-1.82 (m, 5H), 2.47 (s, 3H), 2.80-2.88 (m, 4H), 4.48 (d, 2H, J= 6.02 Hz), 6.73 (d, 1H, J=3.8 Hz), 7.06 (d, 1H, J= 3.8 Hz), 7.32 (d, 1H, J=8.5 Hz), 7.35 (dd, 1H, J= 2.0, 8.5 Hz), 7.48(d, 1H, J= 2.0 Hz), 7.63 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) δ=9.7, 23.3, 24.1, 25.4, 57.1, 60.9, 74.8, 92.8, 128.0, 128.3, 130.4, 130.8, 131.7, 133.8, 135.8, 136.5, 159.8. ESI-MS (MeOH): m/z calcd. 502.1 [M + H]⁺, trovato.

1-(2,4-diclorofenil)-5-(5-(5-idrossipent-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (2c).



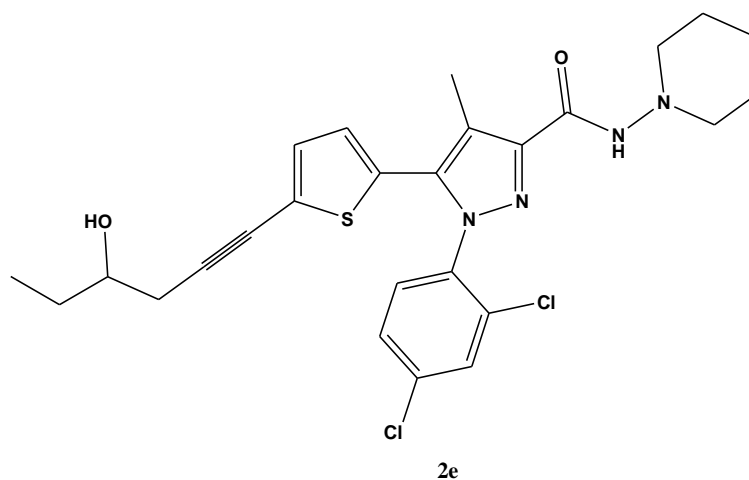
La procedura generale **II** è stata usata per convertire **10** e 4-pentin-1-olo in **2c**. Il prodotto grezzo è stato purificato mediante flash chromatografia (CH₂Cl₂/MeOH, 98:2) Resa 97%. Solido giallo $R_f = 0.31$ (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ =1.39-1.45 (m, 2H), 1.70-1.76 (m, 5H), 1.79-1.84 (m, 2H), 2.45 (s, 3H), 2.53 (t, 2H, J= 6.99 Hz), 2.83 (bs, 4H), 3.76 (t, 2H, J= 6.1 Hz), 6.68 (d, 1H, J=3.7 Hz), 6.95 (d, 1H, J= 3.7 Hz), 7.31 (d, 1H, J=8.6 Hz), 7.35 (dd, 1H, J= 2.0, 8.6 Hz), 7.47 (d, 1H, J= 2.0 Hz), 7.59 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) δ =9.4, 14.2, 16.2, 18.8, 23.3, 25.4, 31.1, 57.1, 61.5, 73.4, 95.7, 119.3, 126.3, 127.9, 128.2, 128.8, 130.3, 130.8, 131.2, 133.7, 135.8, 136.4, 137.3, 144.3, 159.8. ESI-MS (MeOH): m/z calcd. 517.12 [M + H]⁺, trovato 517.1.

1-(2,4-diclorofenil)-5-(5-(6-idrossies-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (2d).



La procedura generale **II** è stata usata per convertire **10** e 5-esenil-1-ol in **2d**. Il prodotto grezzo è stato purificato mediante flash cromatografia (CH₂Cl₂/MeOH, 98:2) Resa 93% Olio giallo. $R_f = 0.32$ (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ =1.33-1.45 (m, 2H), 1.62-1.76 (m, 8H), 2.42-2.46 (m, 5H), 2.84 (bs, 4H), 3.68 (t, 2H, J= 6.0 Hz), 6.68 (d, 1H, J=3.7 Hz), 6.95 (d, 1H, J= 3.7 Hz), 7.31 (d, 1H, J=8.3 Hz), 7.35 (dd, 1H, J= 2.0, 8.6 Hz), 7.48 (d, 1H, J= 2.0 Hz), 7.59 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) δ =10.0, 19.8, 23.7, 25.0, 25.7, 32.1, 57.4, 62.7, 73.6, 96.5, 119.6, 126.7, 128.3, 128.6, 129.1, 130.6, 131.1, 131.4, 134.1, 136.1, 136.7, 137.8, 144.7, 160.1.

1-(2,4-diclorofenil)-5-(5-(4-idrossies-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (2e).

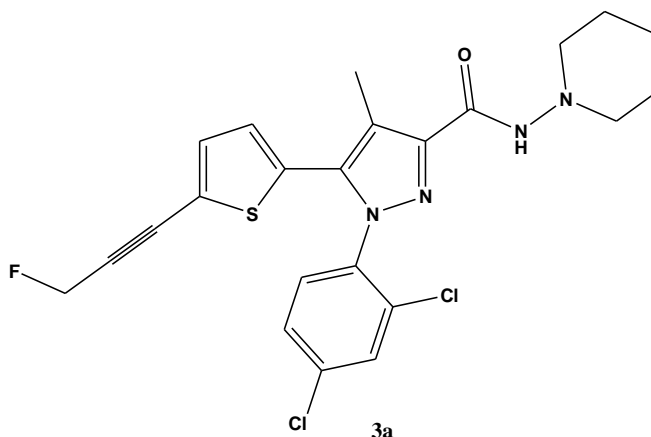


La procedura generale **II** è stata usata per convertire 10 e 5-esin-3-olo in **2e**. Il prodotto grezzo viene purificato mediante cromatografia flash (CH₂Cl₂/MeOH, 98:2) viene ottenuto **2e** (0.530g, yield 96%) come olio marroncino. R_f = 0.56 (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ =1.38-1.45 (m, 2H), 1.54-1.62 (m, 2H), 1.70-1.78 (m, 4H), 1.99 (s, 1H), 2.46 (s, 3H), 2.52-2.67 (m, 2H), 2.83 (bs, 4H), 3.70-3.76 (m, 1H), 6.68 (d, 1H, J=3.7 Hz), 6.99 (d, 1H, J= 3.7 Hz), 7.32 (d, 1H, J=8.1 Hz), 7.35 (dd, 1H, J= 2.2, 8.1 Hz), 7.48 (d, 1H, J= 2.2 Hz), 7.60 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) δ =9.65, 9.9, 23.3, 25.4, 28.2, 29.3, 57.1, 71.4, 75.1, 92.7, 119.3, 125.9, 128.0, 128.2, 129.1, 130.3, 130.8, 131.5, 133.7, 135.8, 136.5, 137.2, 144.3, 159.8. ESI-MS (MeOH): m/z calcd. 531.13 [M + H]⁺, trovato 531.1.

Procedura Generale I: 5-fluoroalchinil Carbossamidi (3a-e).

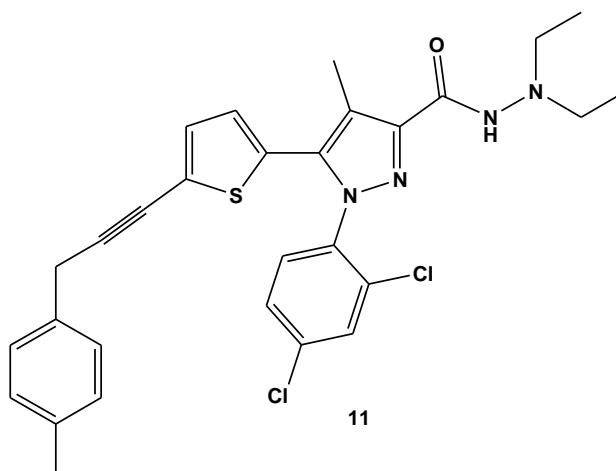
Sotto un flusso di azoto gassoso, in un pallone a tre colli a fondo tondo è stato caricato con una soluzione al 50% di DeoxoFluor in toluene (0.228ml, 0,62 mmoli di DeoxoFluor) diluito con di CH₂Cl₂ anidro (2 ml). La soluzione è stata raffreddata a -78 °C e le 5-idrossialchinil carbossammidi **1a-e** (0,156 g, 0,31 mmoli) in CH₂Cl₂ (0,5 ml) sono state aggiunte goccia a goccia. La miscela così ottenuta è stata agitata alla stessa temperatura per 1 ora, è stato versato in una soluzione di NaHCO₃ satura ed estratta con CH₂Cl₂. La fase organica è stata lavata una volta con HCl 1N (5 ml) e due volte con acqua, poi anidificate con Na₂SO₄ e concentrata sotto pressione ridotta. Il prodotto analiticamente puro è stato isolato mediante flash cromatografia seguita da TLC o RPHPLC.

1-(2,4-diclorofenil)-5-(5-(3-fluoroprop-1-inil)tiiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazolo-3-carbossamide (3a).



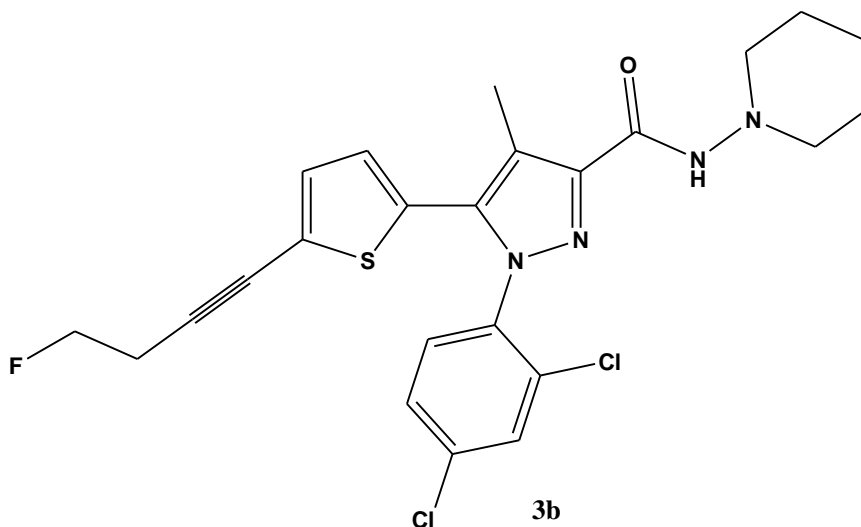
La procedura generale **I** è stata usata per convertire **2a** in **3a**. Il prodotto grezzo è stato purificato mediante flash cromatografia (Esano/AcOEt, 6:4→1:1) Resa= 7%. Olio giallo. R_f = 0.39 (Etere di petrolio/AcOEt, 1:1); ^1H NMR (400MHz, CDCl_3) δ =1.40-1.47 (m, 2H), 1.71-1.80 (m, 4H), 2.48 (s, 3H), 2.88 (bs, 4H), 5.15 (d, 2H, J = 47.7 Hz), 6.76 (d, 1H, J =3.7 Hz), 7.14 (dd, 1H, J = 1.5, 3.7 Hz), 7.33 (dd, 1H, J =0.5, 8.5 Hz), 7.36 (dd, 1H, J = 2.0, 8.5 Hz), 7.49(dd, 1H, J = 0.5, 2.0 Hz), 7.63 (bs, 1H); ^{13}C NMR (100MHz, CDCl_3) δ =9.8, 23.5, 25.1, 57.2, 70.2, 71.8, 71.9, 85.3 (splitted), 119.75, 123.8, 128.2, 128.4, 130.5, 130.9, 131.5, 133.4 (splitted), 135.8, 136.8, 137.1, 144.6, 159.9; ^{19}F NMR (376.5 MHz, CDCl_3) δ = -213.79 (s, 1F). ESI-MS (MeOH): m/z calcd. 491.08 $[\text{M}+\text{H}]^+$, trovato 491.0.

1-(2,4-diclorofenil)-4-metil-N-(piperidin-1-il)-5-(3-p-tolilprop-1-inil)tiofen-2-il)-1H-pirazolo-3-carbossamide (11).



Il composto **11** è stato ottenuto utilizzando la procedura generale **I**. Olio giallo (Resa 15%). R_f = 0.48 (Esano/AcOEt, 1:1); ^1H NMR (400MHz, CDCl_3) δ =1.41-1.46 (m, 2H), 1.71-1.78 (m, 4H), 2.32 (s, 3H), 2.47 (s, 3H), 2.87 (bs, 4H), 3.76 (s, 2H), 6.70 (d, 1H, J =3.8 Hz), 7.00 (d, 1H, J = 3.8 Hz), 7.12 (d, 2H, J =7.8 Hz), 7.22 (d, 2H, J = 7.8 Hz), 7.31 (d, 1H, J = 8.5 Hz), 7.34 (dd, 1H, J = 2.0, 8.5 Hz), 7.48 (d, 1H, J =2.0 Hz), 7.61 (bs, 1H); ^{13}C NMR (100MHz, CDCl_3) δ =9.6, 21.0, 23.3, 25.3, 25.6, 57.0, 74.7, 94.1, 119.3, 127.8, 128.0, 128.3, 129.0, 129.3, 130.3, 130.8, 131.4, 132.9, 133.7, 135.7, 136.5, 159.8; ^{19}F NMR (376.5 MHz, CDCl_3) δ = -213.79 (s, 1F). ESI-MS (MeOH): m/z calcd. 563.14 $[\text{M}+\text{H}]^+$, trovato 563.2.

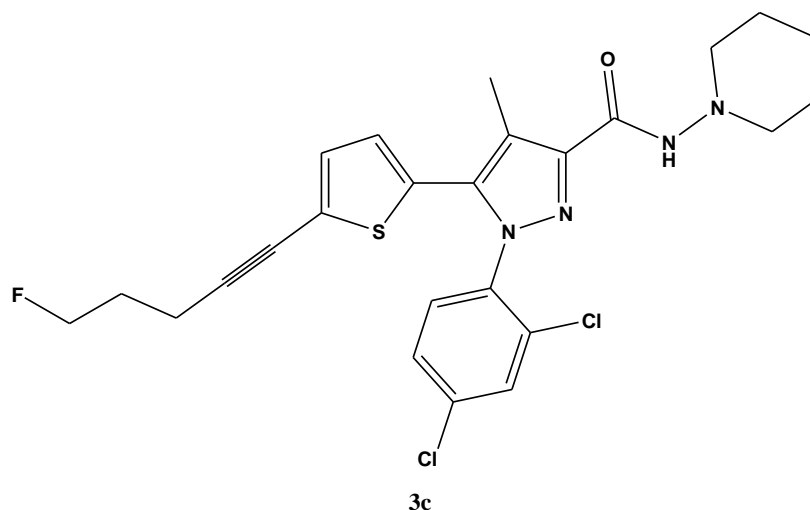
1-(2,4-diclorofenil)-5-(5-(4-fluorobut-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazole-3-carbossamide (3b).



La procedura generale **I** è stata usata per convertire **2b** in **3b**. Il prodotto grezzo è stato purificato mediante flash cromatografia (Esano/AcOet, 6:4→1:1) Olio giallo. Resa 3%. R_f = 0.36 (Esano/AcOEt, 1:1); ^1H NMR (400MHz, CDCl_3) δ =1.42-1.47 (m, 2H), 1.71-1.85 (m, 4H), 2.48 (s, 3H), 2.79-3.03 (m, 6H), 4.56 (dt, 2H, J = 46.7 Hz), 6.71 (d, 1H, J =3.8 Hz), 7.02 (d, 1H, J = 3.8 Hz), 7.33 (d, 1H, J = 8.5 Hz), 7.36 (dd, 1H, J = 2.0, 8.5 Hz), 7.50(d, 1H, J = 2.0 Hz), 7.67 (bs, 1H); ^{19}F

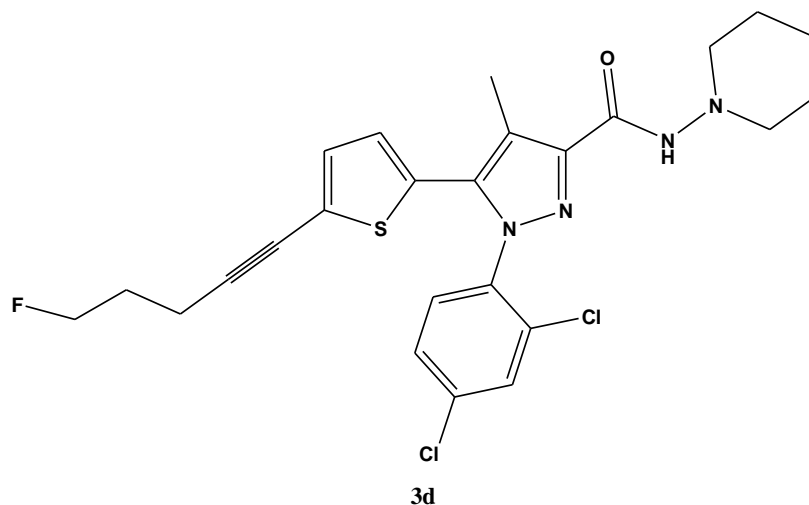
NMR (376.5 MHz, CDCl₃) δ = -215.26 (s, 1F). ESI-MS (MeOH): m/z calcd. 505.10 [M+H]⁺, trovato 505.1.

1-(2,4-diclorofenil)-5-(5-(6-fluoroes-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (3c).



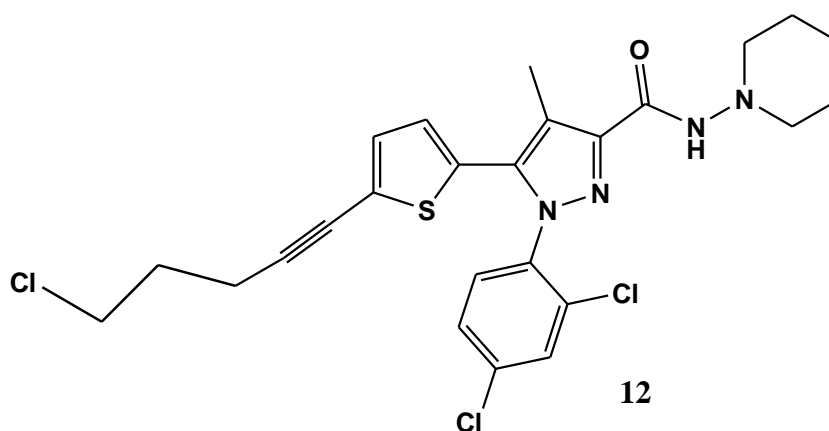
La procedura generale **I** è stata usata per convertire **2c** nel prodotto **3c**. Il prodotto grezzo è stato purificato mediante flash cromatografia (Esano/AcOet, 6:4→1:1). Si ottiene un olio bianco dopo una purificazione con HPLC, colonna semi-preparativa, eluente: A: H₂O, eluente B: CH₃CN; in condizioni isocratiche Resa 7%. R_f = 0.51 (Esano/AcOEt, 1:1); ¹H NMR (400MHz, CDCl₃) δ =1.40-1.47 (m, 2H), 1.69-1.91 (m, 8H), 2.45-2.52 (m, 5H), 2.81-2.92 (m, 4H), 4.49 (dt, 2H, J= 5.8, 47.2 Hz), 6.68 (d, 1H, J=3.8 Hz), 6.98 (d, 1H, J= 3.8 Hz), 7.33 (dd, 1H, J=0.5, 8.5 Hz), 7.36 (dd, 1H, J= 2.0, 8.5 Hz), 7.49 (dd, 1H, J= 0.5, 2.0 Hz), 7.60 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) δ =9.8, 19.5, 23.5, 24.4 (splitted), 25.6, 29.7 (splitted), 57.3, 83.7 (splitted), 95.92, 119.5, 126.4, 128.1, 128.4, 129.0, 130.5, 131.0, 131.3, 133.9, 136.0, 136.6, 137.5, 144.6, 160.0; ¹⁹F NMR (376.5 MHz, CDCl₃) δ = -218.93 (s, 1F). ESI-MS (MeOH): m/z calcd. 533.13 [M+H]⁺, trovato 533.1.

1-(2,4-diclorofenil)-5-(5-(5-fluoropent-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (3d)



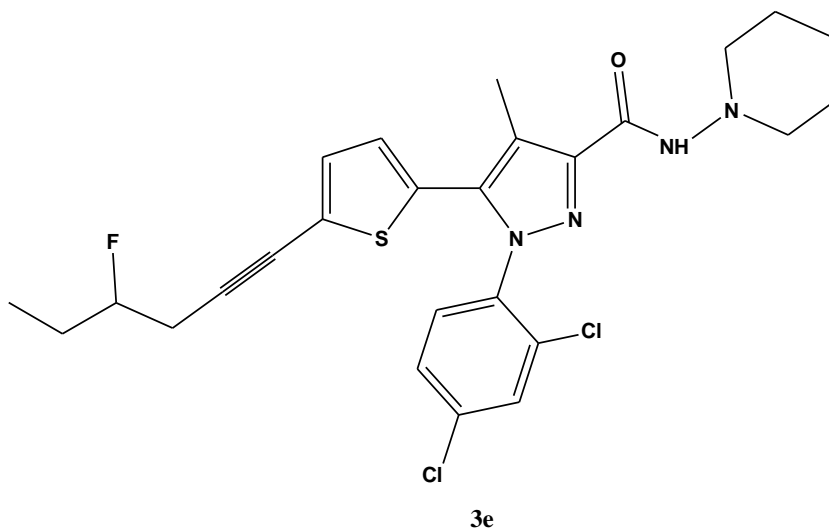
La procedura generale I è stata usata per convertire 2d nel prodotto 3d. Il prodotto grezzo viene purificato mediante flash cromatografia (CH₂Cl₂/MeOH, 98:2) Resa 93% .Olio giallo. R_f = 0.32 (CH₂Cl₂/MeOH, 98:2); ¹H NMR (400MHz, CDCl₃) δ=1.33-1.45 (m, 2H), 1.62-1.76 (m, 8H), 2.42-2.46 (m, 5H), 2.84 (bs, 4H), 3.68 (t, 2H, J= 6.0 Hz), 6.68 (d, 1H, J=3.7 Hz), 6.95 (d, 1H, J= 3.7 Hz), 7.31 (d, 1H, J=8.3 Hz), 7.35 (dd, 1H, J= 2.0, 8.6 Hz), 7.48 (d, 1H, J= 2.0 Hz), 7.59 (bs, 1H); ¹³C NMR (100MHz, CDCl₃) =10.0, 19.8, 23.7, 25.0, 25.7, 32.1, 57.4, 62.7, 73.6, 96.5, 119.6, 126.7, 128.3, 128.6, 129.1, 130.6, 131.1, 131.4, 134.1, 136.1, 136.7, 137.8, 144.7, 160.1.

1-(2,4-diclorofenil)-5-(5-(6-cloro-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (12).



Il composto **12** è stato ottenuto utilizzando la procedura generale **I**. Il prodotto grezzo è stato purificato mediante flash cromatografia (Esano/AcOEt, 6:4→1:1) Si ottiene un olio bianco dopo una purificazione con HPLC , colonna semi-preparativa, eluente: A: H₂O, eluente B: CH₃CN; in condizioni isocratica. Resa 14%. R_f = 0.51 (Esano/AcOEt, 1:1); ¹H NMR (400MHz, CDCl₃) δ =1.42-1.49 (m, 2H), 1.72-1.85 (m, 6H), 1.90-1.99 (m, 2H), 2.45-2.52 (m, 5H), 2.97 (bs, 4H), 3.60 (t, 2H, J= 6.5 Hz), 6.70 (d, 1H, J=3.8 Hz), 6.98 (d, 1H, J= 3.8 Hz), 7.33 (dd, 1H, J=0.5, 8.5 Hz), 7.36 (dd, 1H, J= 2.0, 8.5 Hz), 7.50 (dd, 1H, J= 0.5, 2.0 Hz), 7.60 (bs, 1H); ESI-MS (MeOH): m/z calcd. 551.09 [M+H]⁺, trovato 551.0.

1-(2,4-diclorofenil)-5-(5-(4-fluorohex-1-inil)tiofen-2-il)-4-metil-N-(piperidin-1-il)-1H-pirazol-3-carbossamide (3e).



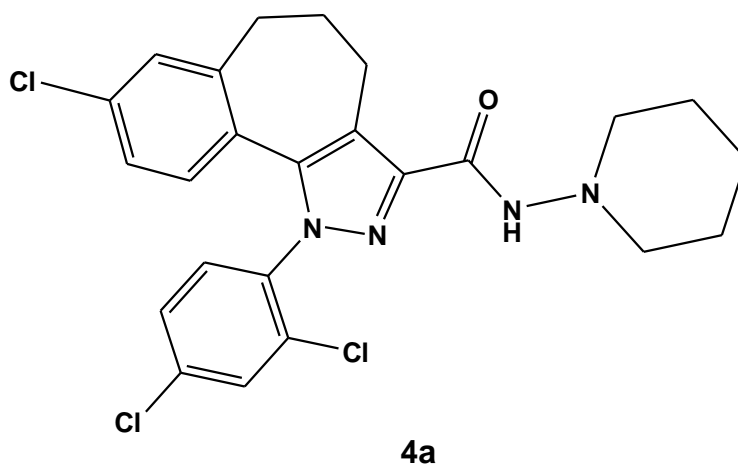
La procedura generale **I** è stata usata per convertire **2e** nel prodotto **3e**. Il prodotto grezzo è stato purificato mediante flash cromatografia (Esano/AcOEt, 6:4→1:1) Si ottiene un olio giallo dopo una purificazione con HPLC , colonna semi-preparativa, eluente: A: H₂O, eluente B: CH₃CN; in condizioni isocratica. R_f = 0.16 (Esano/AcOEt, 1:1); ¹H NMR (400MHz, CDCl₃) δ =1.40-1.47 (m, 2H), 1.68-1.83 (m, 6H), 2.46 (s, 3H), 2.78 (dd, 2H, J= 3.0, 6.1, 18.5 Hz), 2.89 (bs, 4H), 4.60 (dq, 1H, J= 6.1, 48.0 Hz), 6.89 (d, 1H, J=3.8 Hz), 7.01 (d, 1H, J= 3.8 Hz), 7.33 (dd, 1H, J=0.5, 8.5 Hz), 7.36 (dd, 1H, J= 2.0, 8.5 Hz), 7.49 (dd, 1H, J= 0.5, 2.0 Hz), 7.66 (bs, 1H); ¹⁹F NMR (376.5 MHz, CDCl₃) δ = -178.97 (s, 1F).

Procedure sintetiche dettagliati intermedi e prodotti finali della terza serie

Procedura generale A: preparazione di carboidrazidi 4a-c e carbossamidi 4d-i.

Una miscela dell'appropriato acido carbossilico **24** o **25** (1.0 eq, 0.25 mmol), 1-idrossibenzotriazolo idrato (HOBt) (1.2 eq, 0.30 mmol) e N-(3-dimetilaminopropil)-N'-etilcarbodiimide idrocloride (EDC) (1.2 eq, 0.30 mmol) in diclorometano (2.0 mL) è stato lasciato reagire a temperatura ambiente per 1 h. Una soluzione dell'opportuna idrazina o amina (2.0 eq, 0.50 mmol) in diclorometano (3.0 mL) viene aggiunta goccia a goccia. La miscela risultante viene lasciata reagire a temperatura ambiente per 22 h. Il solvente viene allontanato per evaporazione sotto vuoto e il grezzo viene purificato con la flash-cromatografia per ottenere il composto desiderato. Quando vengono utilizzate l'idrazina o l'ammina come sali cloridati, viene aggiunta alla miscela trietilamina (TEA) (2.0 eq, 0.50 mmol).

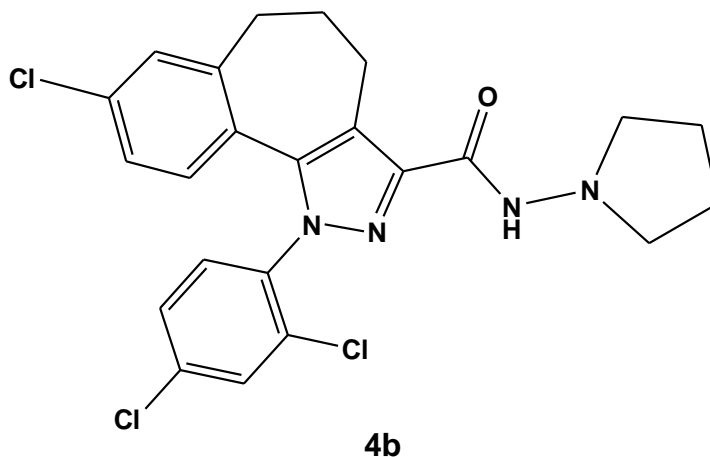
N-Piperidin-1-il-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita [1,2-c]pirazolo-3-carbossamide (4a)



Solido bianco; Resa: 79%; mp 199-200°C (acetone), (202 °C [1]); R_f 0.40 (dietil eter/etere di petrolio, 6:4); IR 3200, 1650; ESI-MS (*m/z*): 513.1 (M+Na);

^1H -NMR (CDCl_3) δ 1.40-1.48 (m, 2H), 1.72-1.80 (m, 4H), 2.19-2.30 (m, 2H), 2.66 (t, 2H, $J = 6.6$ Hz), 2.83-2.90 (m, 4H), 2.90-3.48 (m, 2H), 6.56 (d, 1H, $J = 8.3$ Hz), 7.00 (dd, 1H, $J = 2.2$ and 8.3 Hz), 7.30 (d, 1H, $J = 2.2$ Hz), 7.40 (dd, 1H, $J = 2.2$ and 8.3 Hz); ^{13}C -NMR (CDCl_3) δ 20.2, 23.4, 25.5, 31.4, 32.6, 57.1, 122.7, 126.2, 127.7, 128.0, 128.2, 129.9, 130.4, 130.5, 132.5, 134.2, 135.9, 136.0, 142.2, 143.6, 143.8, 160.0. Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_3\text{N}_4\text{O}$ (C, H, Cl, N).

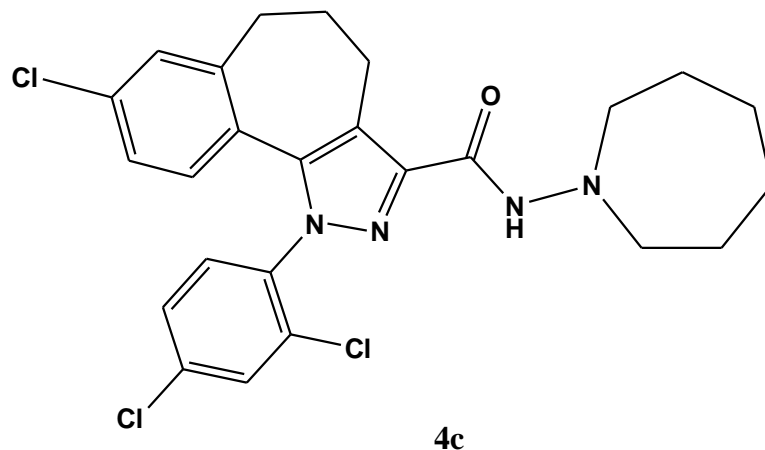
N-Pirrolidin-1-il-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (4b)



A causa di un eccesso di sale cloridrato, sono stati utilizzati in questa reazione 2 eq di TEA. Solido bianco; Resa: 60%; mp 127-128°C; Rf 0.33 (etere di petrolio/EtOAc 4:6);-NMR (CDCl_3) δ 1.82-1.96 (m, 4H), 2.25 (t, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 6.4$ Hz), 2.90-3.10 (m, 6H), 6.57 (d, 1H, $J = 8.2$ Hz), 6.99 (dd, 1H, $J_o = 8.2$ Hz, $J_m = 2.2$ Hz), 7.28-7.31 (m, 1H), 7.37-7.49 (m, 3H), 7.66 (br s, 1H, NH exch. with D_2O); ^{13}C -NMR (CDCl_3) δ 20.04 (CH_2), 22.20 ($\text{CH}_2 \times 2$), 31.32 (CH_2), 32.41 (CH_2), 55.31 ($\text{CH}_2 \times 2$), 122.47 (C), 126.10 (CH), 127.50 (C), 127.93 (CH), 128.11 (CH), 129.81 (CH), 130.29 (CH), 130.37 (CH), 132.36 (C), 134.06 (C), 135.84 (C), 142.13 (C), 143.34 (C), 143.67 (C), 160.72 (C), 169.98 (CO); API-ES calcd for 475.8, trovato 475.10; Anal. calcd for $\text{C}_{23}\text{H}_{21}\text{Cl}_3\text{N}_4\text{O}$: C,

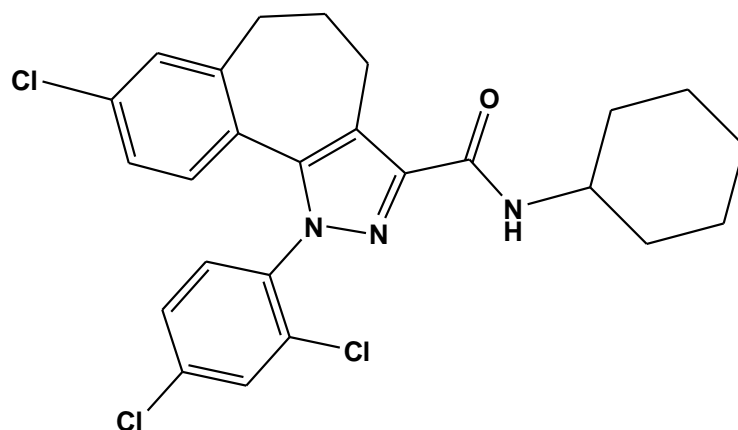
58.06; H, 4.45; Cl, 22.35; N, 11.78. Trovato: C, 58.25; H, 4.46; Cl, 22.37; N, 11.81.

N-(azepan-1-il)--8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazol-3-carbossamide (4c)



Solido bianco; Resa: 35%; mp 160-161°C; R_f 0.54 (etere di petrolio/EtOAc 6:4); IR 1659, 3174; ¹H-NMR (CDCl₃) δ 1.60-1.80 (m, 10H), 2.25 (qu, 2H, *J* = 6.0 Hz), 2.65 (t, 2H, *J* = 6.2 Hz), 3.15 (t, 4H, *J* = 5.6 Hz), 6.56 (d, 1H, *J* = 8.4 Hz), 6.99 (dd, 1H, *J_o* = 8.6 Hz, *J_m* = 1.8 Hz), 7.30 (s, 1H), 7.37-7.48 (m, 3H), 8.10 (br s, 1H, NH exch. with D₂O); ¹³C-NMR (CDCl₃) □ 20.10 (CH₂), 26.36 (CH₂), 26.95 (CH₂ x 2), 29.67 (CH₂), 31.41 (CH₂), 32.49 (CH₂), 58.32 (CH₂ x 2), 122.57 (C), 126.17 (CH), 127.62 (C), 127.10 (CH), 128.14 (CH), 129.89 (CH), 130.32 (CH), 130.46 (CH), 132.42 (C), 134.11 (C x 2), 135.86 (C), 142.17 (C), 143.74 (C x 2), 160.72 (CO); API-ES calcd for 503.9, trovato 503.01; Anal. calcd for C₂₅H₂₅Cl₃N₄O: C, 59.59; H, 5.00; Cl, 21.10; N, 11.12. Trovato: C, 59.76; H, 5.02; Cl, 21.15; N, 11.13.

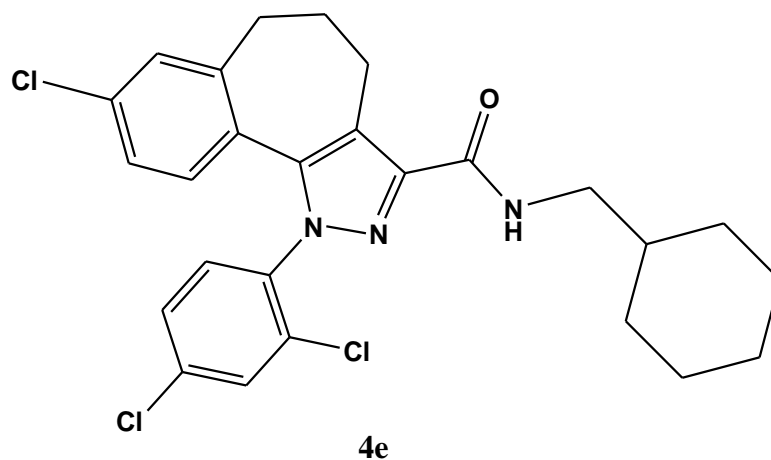
N-Cicloesil-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (4d)



4d

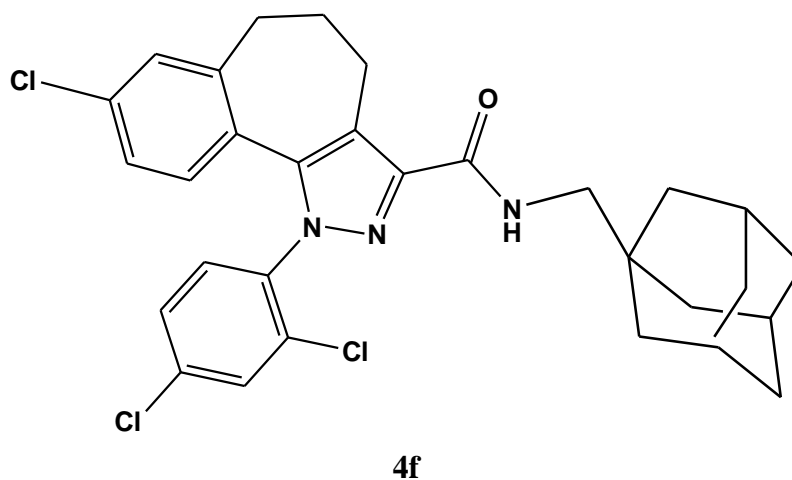
Preparata mediante procedura generale I per convertire 24a e cicloesilamina nel prodotto 4d. Solido bianco; Resa: 50%; mp 96-98°C; Rf 0.54 (etere di petrolio/EtOAc 8:2); IR 1633, 3201; $^1\text{H-NMR}$ (CDCl_3) δ 1.16-1.45 (m, 7H), 1.62-1.82 (m, 2H), 1.96-2.06 (m, 2H), 2.25 (qu, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 6.6$ Hz), 2.95-3.12 (m, 2H), 6.57 (d, 1H, $J = 8.4$ Hz), 6.86 (d, 1H, $J = 8.6$ Hz), 6.99 (dd, 1H, $J_o = 8.0$ Hz, $J_m = 2.0$ Hz), 7.30 (d, 1H, $J = 2.2$ Hz), 7.38-7.46 (m, 2H), 7.50 (br s, 1H, NH exch. with D_2O); $^{13}\text{C-NMR}$ (CDCl_3) \square 20.18 (CH_2), 25.01 ($\text{CH}_2 \times 2$), 25.59 (CH_2), 31.49 (CH_2), 32.50 (CH_2), 33.19 ($\text{CH}_2 \times 2$), 47.92 (CH), 122.22 (C), 126.16 (CH), 127.74 (C), 127.99 (CH), 128.17 (CH), 129.88 (CH), 130.38 (CH), 130.45 (CH), 132.46 (C), 134.05 (C), 135.85 (C), 135.98 (C), 142.28 (C), 143.73 (C), 144.25 (C), 161.77 (CO); API-ES calcd for 488.84, trovato 488.10; Anal. calcd for $\text{C}_{25}\text{H}_{24}\text{Cl}_3\text{N}_3\text{O}$: C, 61.43; H, 4.95; Cl, 21.76; N, 8.60. Trovato: C, 61.25; H, 4.94; Cl, 21.69; N, 8.58.

N-Ciclohesilmetil-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita [1,2-c]pirazolo-3-carbossamide (4e).



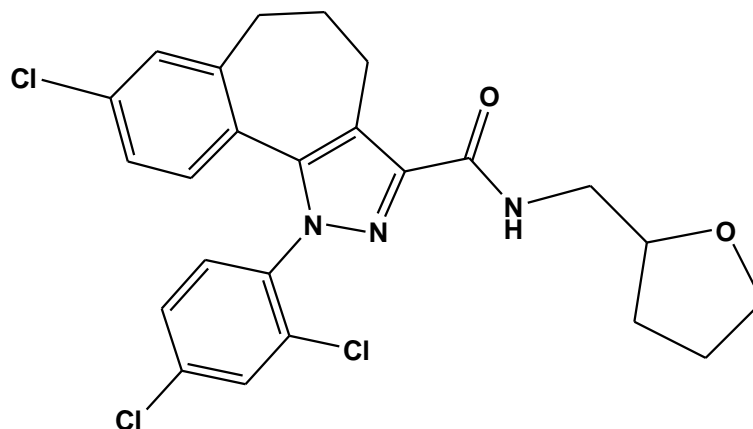
Solido bianco; Resa: 44%; R_f = 0.45 (etere di petrolio/EtOAc 9:1); IR 3410, 1670; $^1\text{H-NMR}$ (CDCl_3) δ 0.92-1.03 (m, 2H); 1.10-1.32 (m, 4H); 1.53-1.84 (m, 5H); 2.20-2.31 (m, 2H); 2.60-2.72 (m, 3H); 2.80-3.15 (m, 1H); 3.20-3.30 (m, 2H); 6.58 (d, 1H, J = 8.0 Hz); 6.97-7.08 (m, 2H); 7.30 (d, 1H, J = 1.6 Hz); 7.40 (dd, 1H, J = 1.7 and 8.4 Hz); 7.42-7.48 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) \square 20.2, 25.9, 26.4, 31.0, 31.5, 32.5, 38.1, 45.3, 122.2, 126.2, 127.8, 128.1, 128.2, 129.9, 130.4, 130.5, 132.5, 134.1, 135.9, 136.1, 142.3, 143.8, 144.2, 162.7. Anal. calc. for $\text{C}_{26}\text{H}_{26}\text{Cl}_3\text{N}_3\text{O}$: C, 62.10; H, 5.21; Cl, 21.15; N, 8.36. Trovato: C, 62.03; H, 5.20; Cl, 21.13; N, 8.34.

N-(1-Adamantilmetil)-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloep[1,2-c]pirazolo-3-carbossamide (4f).



Solido bianco; Resa: 59%; $R_f = 0.41$ (etere di petrolio/EtOAc 9:1); IR 3405, 1669; $^1\text{H-NMR}$ (CDCl_3) δ 1.57 (bs, 6H); 1.61-1.75 (m, 6H); 1.95-2.02 (m, 3H); 2.20-2.30 (m, 2H); 2.63-2.73 (m, 3H); 2.82-3.18 (m, 3H); 6.58 (d, 1H, $J = 8.4$ Hz); 6.97-7.09 (m, 2H); 7.30 (bs, 1H); 7.40 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.44-7.50 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) \square 20.3, 28.3, 31.4, 32.5, 34.1, 37.0, 40.3, 50.5, 122.2, 126.2, 127.8, 128.1, 128.2, 129.9, 130.4, 130.5, 132.5, 134.1, 135.9, 136.1, 142.3, 143.8, 144.3, 162.9. Anal. calc. for $\text{C}_{30}\text{H}_{30}\text{Cl}_3\text{N}_3\text{O}$: C, 64.93; H, 5.45; Cl, 19.17; N, 7.57. Trovato: C, 64.81; H, 5.44; Cl, 19.10; N, 7.55.

N-(2,3,4,5-Tetraidrofurfuril)-8-chloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo [6,7] cicloepta[1,2-c]pirazole-3-carbossamide (4g).

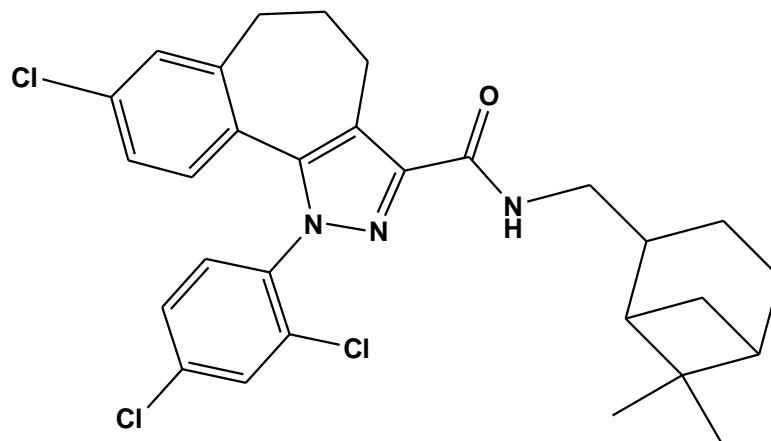


4g

Solido bianco; Resa: 54%; $R_f = 0.38$ (etere di petrolio/EtOAc 7:3); IR 3409, 1667; $^1\text{H-NMR}$ (CDCl_3) \square 1.58-1.74 (m, 1H); 1.84-1.96 (m, 2H); 1.97-2.08 (m, 1H); 2.19-2.31 (m, 2H); 2.61-2.72 (m, 2H); 2.75-3.28 (m, 2H); 3.31-3.41 (m, 1H); 3.66-3.81 (m, 2H); 3.84-3.94 (m, 1H); 4.04-4.12 (m, 1H); 6.57 (d, 1H, $J = 8.0$ Hz); 7.00 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.27-7.32 (m, 2H); 7.39 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.41-7.48 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) \square 19.7, 25.3, 28.2, 30.9, 31.9, 42.2, 67.6, 77.3, 121.6, 125.6, 126.4, 127.2, 127.5, 127.6, 129.3, 129.8, 129.9, 131.8, 133.5, 135.2, 141.6, 143.2, 143.4, 162.3. Anal. calc. for

C₂₄H₂₂Cl₃N₃O₂: C, 58.73; H, 4.52; Cl, 21.67; N, 8.56. Trovato: C, 58.69; H, 4.51; Cl, 21.65; N, 8.54.

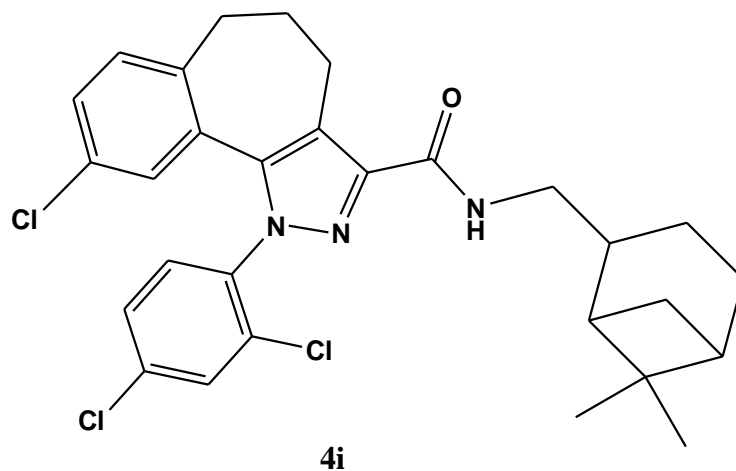
N-(Mirtanil)-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita [1,2-c]pirazolo-3-carbossamide (4h).



4h

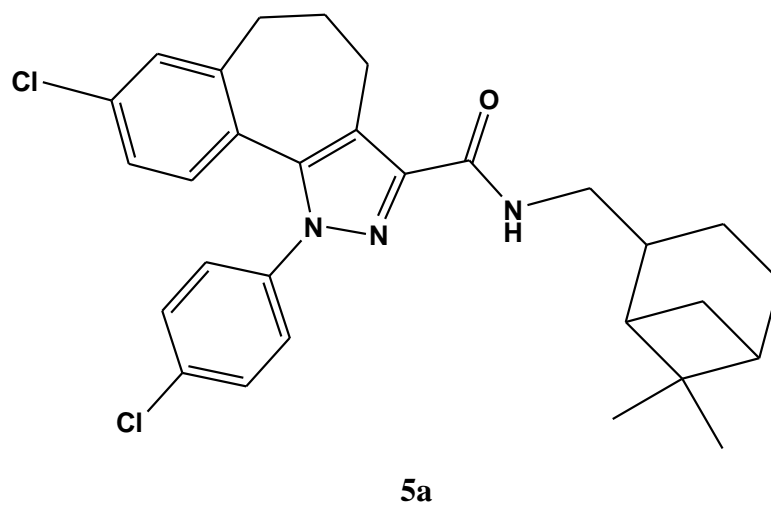
Solido bianco; Resa: 81%; R_f = 0.48 (etere di petrolio/EtOAc 6:4); IR 3400, 1662; ¹H-NMR (CDCl₃) δ 1.07 (s, 3H); 1.21 (s, 3H); 1.52-1.63 (m, 5H); 1.82-2.05 (m, 4H); 2.15-2.29 (m, 4H); 2.64 (t, 2H, *J* = 6.6 Hz); 3.35-3.45 (m, 2H); 6.59 (d, 1H, *J* = 8.0 Hz); 7.00 (dd, 1H, *J* = 2.2 e 8.0 Hz); 7.25-7.32 (m, 1H); 7.37-7.43 (m, 3H); 7.51 (bt, 1H, *J* = 5.4 Hz); ¹³C-NMR (CDCl₃) □ 19.86 (CH₃), 20.20 (CH₃), 23.23 (CH₂), 26.02 (CH₂), 27.96 (CH₂), 31.44 (CH₂), 33.49 (CH₂), 33.28 (CH₂), 38.70 (C), 41.32 (CH), 41.48 (CH), 43.81 (CH), 44.58 (CH₂), 122.15 (C), 126.16 (CH), 127.68 (CH), 128.01 (CH), 128.15 (CH), 129.88 (CH), 130.33 (CH), 130.45 (CH), 132.45 (C), 134.05 (C), 135.85 (C), 135.97 (C), 142.27 (C), 143.72 (C), 144.15 (C), 162.61 (C=O). Anal. calc. for C₂₉H₃₀Cl₃N₃O: C, 64.15; H, 5.57; Cl, 19.59; N, 7.74. Trovato: C, 64.09; H, 5.55; Cl, 19.56; N, 7.72.

N-(Mirtanil)-9-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita [1,2-c]pirazolo-3-carbossamide (4i).



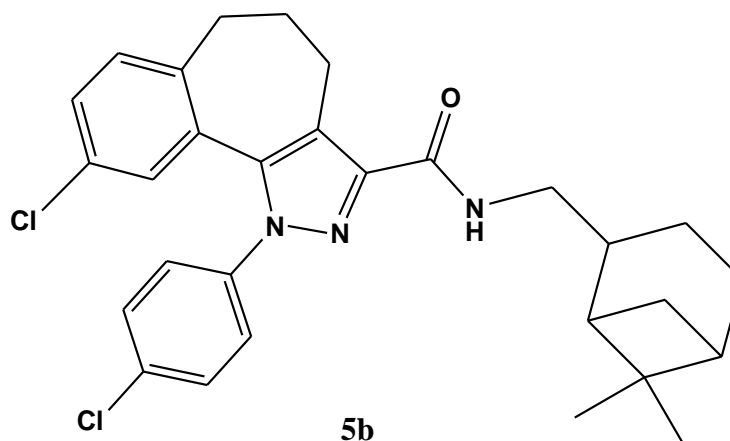
Solid bianco; Resa: 87%; R_f = 0.50 (etere di petrolio/EtOAc 6:4); IR 3405, 1664; ¹H-NMR (CDCl₃) δ 1.08 (s, 3H); 1.20 (s, 3H); 1.53-1.61 (m, 5H); 1.84-2.03 (m, 4H); 2.19-2.30 (m, 4H); 2.63 (t, 2H, *J* = 6.4 Hz); 3.37-3.46 (m, 2H); 6.61 (s, 1H); 6.93 (bt, 1H, *J* = 5.5 Hz); 6.98-7.10 (m, 1H); 7.17-7.23 (m, 2H); 7.49-7.55 (m, 2H); ¹³C-NMR (CDCl₃) □ 19.3, 19.6, 22.6, 25.5, 27.4, 31.0, 31.5, 32.7, 38.1, 40.8, 41.0, 43.3, 44.0, 122.0, 126.2, 127.6, 127.8, 129.8, 129.9, 130.2, 130.5, 131.1, 132.0, 135.3, 135.4, 139.7, 141.5, 143.6, 162.0. Anal. calc. for C₂₉H₃₀Cl₃N₃O: C, 64.15; H, 5.57; Cl, 19.59; N, 7.74. Trovato: C, 64.03; H, 5.56; Cl, 19.57; N, 7.73.

N-(Mirtanil)-8-cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (5a).



Solido bianco; resa: 87%; $R_f = 0.50$ (etere di petrolio/EtOAc 7:3); IR 3405, 1658; $^1\text{H-NMR}$ (CDCl_3) δ 1.08 (s, 3H); 1.21 (s, 3H); 1.50-1.70 (m, 3H); 1.90-2.02 (m, 4H); 2.03-2.50 (m, 9H); 2.83-2.95 (m, 2H); 3.38-3.50 (m, 2H); 5.37 (s, 2H); 6.85-7.31 (m, 8H); $^{13}\text{C-NMR}$ (CDCl_3) \square 19.90 (CH_3), 21.07 (CH_3), 21.35 (CH_3), 23.24 (CH_2), 26.04 (CH_2), 28.01 (CH_2), 30.75 (CH_2), 33.48 (CH_2), 33.27 (CH_2), 38.73 (C), 41.37 (CH), 41.61 (CH), 43.93 (CH), 44.97 (CH_2), 52.09 (CH_2), 115.75 (C), 116.40 (C), 125.65 (CH), 126.72 (CH x 2), 127.13 (CH x 2), 127.38 (CH), 129.55 (CH), 132.25 (C), 133.33 (C), 133.64 (C), 137.61 (C), 138.75 (C), 146.57 (C), 164.70 (C=O). Anal. calc. for $\text{C}_{31}\text{H}_{36}\text{ClN}_3\text{O}$: C, 74.16; H, 7.23; Cl, 7.06; N, 8.37. Trovato: C, 74.00; H, 7.22; Cl, 7.04; N, 8.35.

N-(Mirtanil)-9-cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (5b).

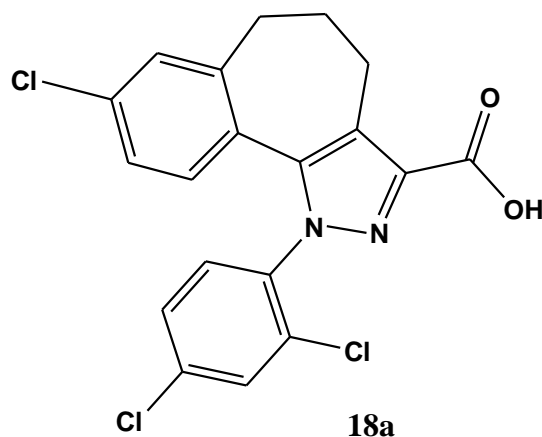


Solido bianco; Resa: 60%; $R_f = 0.48$ (etere di petrolio/EtOAc 7:3); IR 3408, 1659; $^1\text{H-NMR}$ (CDCl_3) δ : 1.15 (s, 3H); 1.19 (s, 3H); 1.90-2.14 (m, 11H); 2.31 (s, 3H); 2.42-2.46 (m, 2H); 2.85-2.89 (m, 2H); 3.39-3.45 (m, 2H); 5.38 (s, 2H); 6.92-7.22 (m, 8H). Anal. calc. for $\text{C}_{31}\text{H}_{36}\text{ClN}_3\text{O}$: C, 74.16; H, 7.23; Cl, 7.06; N, 8.37. Trovato: C, 74.11; H, 7.21; Cl, 7.04; N, 8.36.

Generale procedura per sintesi degli acidi carbossilici 18a, 18b, 19a, e 19b (Procedura A1).

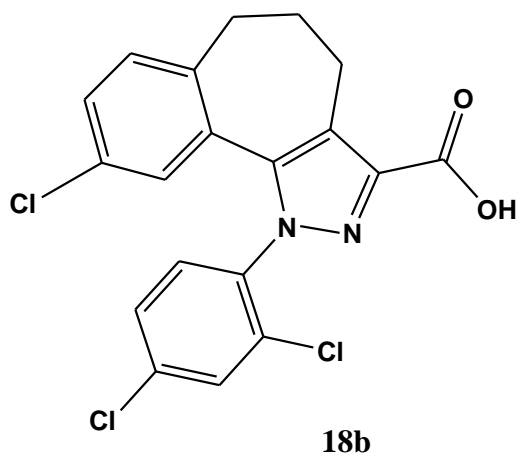
Una miscela di appropriato estere (1.12 mmol) in metanolo (5 mL) è stato aggiunto a una soluzione di idrossido di potassio (0.13 g, 2.25 mmol) in metanolo (4 mL). La risultante miscela viene scaldata a riflusso per una notte. La miscela viene lasciata raffreddare a temperatura ambiente e poi versata in acqua e acidificata con 1 N HCl. Il precipitato è stato filtrato, lavato con acqua ed essiccato per dare i prodotti del titolo.

Sintesi di 8-Cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobzenzo[6,7]cicloeppta[1,2-c]pirazolo-3-carbossilico acido (18a)



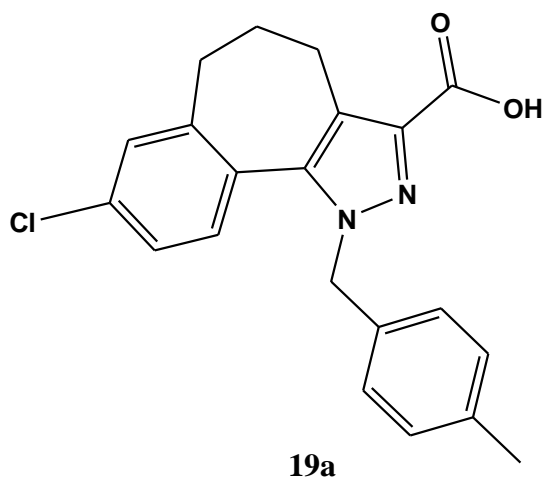
Solido bianco; resa 96%; R_f 0.41 ($\text{CHCl}_3/\text{MeOH}$ 9:1); mp 270°C; IR 1690, 3410; ^1H NMR (CDCl_3) δ 2.25-2.30 (m, 2H), 2.68 (t, 2H, $J = 6.4$ Hz), 3.10-3.23 (m, 2H), 4.50 (bs, 1H), 6.61 (d, 1H, $J = 8.4$ Hz), 7.03 (dd, 1H, $J = 2.2$ and 8.2 Hz), 7.32 (d, 1H, $J = 2.0$ Hz), 7.39-7.44 (m, 2H), 7.52 (d, 1H, $J = 8.0$ Hz). Anal. ($\text{C}_{19}\text{H}_{13}\text{Cl}_3\text{N}_2\text{O}_2$) C, H, Cl, N.

Sintesi di 9-Cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobzenzo[6,7]cicloeppta[1,2-c]pirazolo-3-carbossilico acido (18b).



Solido bianco; Resa 98%; R_f 0.60 ($\text{CHCl}_3/\text{MeOH}$ 9:1); mp 250°C ; IR 1716, 3419; ^1H NMR (CDCl_3) δ 2.25-2.28 (m, 2H), 2.68 (t, 2H, $J = 6.4$ Hz), 3.08-3.30 (m, 2H), 4.80 (br s, 1H, OH exchange with D_2O), 6.63 (d, 1H, $J = 1.8$ Hz), 7.20-7.30 (m, 2H), 7.40-7.52 (m, 2H), 7.59 (d, 1H, $J = 9.0$ Hz). Anal. ($\text{C}_{19}\text{H}_{13}\text{Cl}_3\text{N}_2\text{O}_2$) C, H, Cl, N.

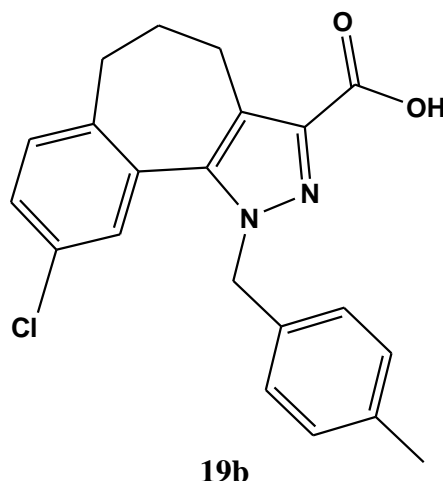
Sintesi di 8-Cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepta[1,2-c]pirazolo-3-carbossilico acido (19a)



Solido bianco; Resa 92%; IR 1716, 3413; ^1H NMR (CDCl_3) δ 2.15-2.35 (m, 2H), 2.38 (s, 3H), 2.67 (t, 2H, $J = 6.8$ Hz), 3.05-3.30 (m, 2H), 3.55 (s, 2H),

6.57 (d, 1H, $J = 8.1$ Hz), 7.00 (dd, 1H, $J = 2.0$ and 8.1 Hz), 7.20 (d, 1H, $J = 2.0$ Hz), 7.22-7.31 (m, 4H). Anal. ($C_{21}H_{19}ClN_2O_2$) C, H, Cl, N.

Sintesi di 9-Cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossilico acido (19b).

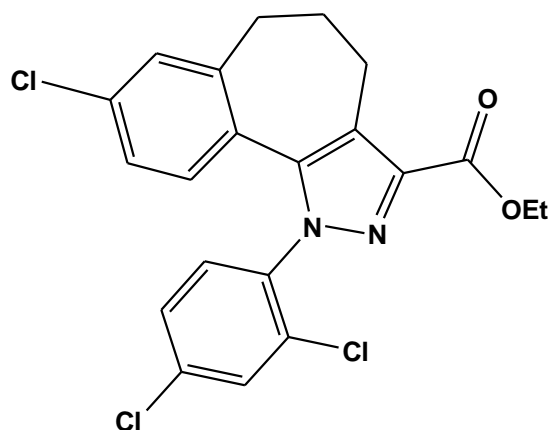


Solido bianco; Resa 96%; R_f 0.36 (etere di petrolio /AcOEt 8.5:1.5); IR 1716, 3420; 1H NMR ($CDCl_3$) δ 2.16-2.36 (m, 2H), 2.38 (s, 3H), 2.65 (t, 2H, $J = 6.8$ Hz), 3.05-3.34 (m, 2H), 3.56 (s, 2H), 6.61 (d, 1H, $J = 8.1$ Hz), 7.00 (dd, 1H, $J = 2.2$ and 8.1 Hz), 7.21 (d, 1H, $J = 2.1$ Hz), 7.24-7.34 (m, 4H). Anal. ($C_{21}H_{19}ClN_2O_2$) C, H, Cl, N.

Generale procedura per la sintesi di etil carbossilato 16a, 16b, 17a, e 17b (Procedura Generale A2).

Una miscela del dichetoestere (0.41 g, 1.39 mmol) e 2,4-diclorofenilidrazina cloridrata o -metilbenzilidrazina cloridrata (1.81 mmol) in EtOH (9 mL) è stata riscaldata a riflusso per 14 h. La miscela viene lasciata raffreddare a temperatura ambiente, e il solvente è stato rimosso a pressione ridotta a dare un solido che è stato purificato mediante flash cromatografia (etere di petrolio/EtOAc, 8.5:1.5) per ottenere il prodotto.

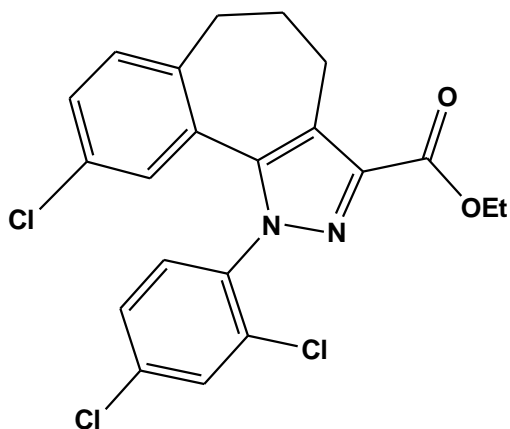
Sintesi di Etil 8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossilato (16a)



16a

Solido giallo; Resa 80%; R_f 0.48 (etere di petrolio/ AcOEt, 8.5:1.5); mp 158-160°C (tritured with petroleum ether), IR 1724; ^1H NMR (CDCl_3) δ 1.43 (t, 3H, $J = 7.2$ Hz), 2.20-2.36 (m, 2H), 2.66 (t, 2H, $J = 6.4$ Hz), 3.10-3.30 (m, 2H), 4.45 (q, 2H, $J = 7.2$ Hz), 6.60 (d, 1H, $J = 8.4$ Hz), 7.02 (dd, 1H, $J = 2.2$ and 8.4 Hz), 7.31 (d, 1H, $J = 1.8$ Hz), 7.37-7.42 (m, 2H), 7.54 (d, 1H, $J = 9.2$ Hz). Anal. ($\text{C}_{21}\text{H}_{17}\text{Cl}_3\text{N}_2\text{O}_2$) C, H, Cl, N.

Sintesi di Etil 9-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo [6,7]cicloepita[1,2-c]pirazolo-3-carbossilato (16b)

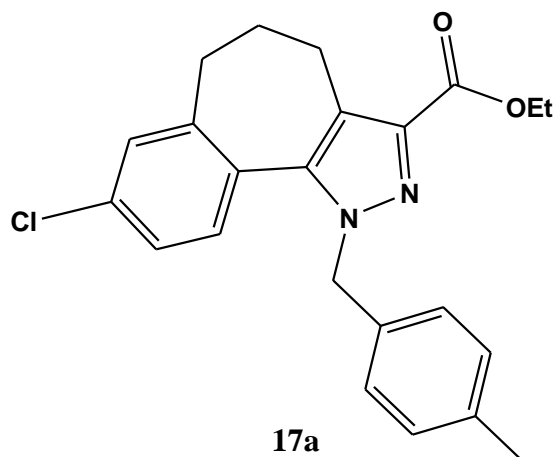


16b

Solido giallo; Resa 70%; R_f 0.43 (etere di petrolio/ AcOEt, 9:1); mp 176-178°C (tritured with petroleum ether), IR 1710; ^1H NMR (CDCl_3) δ 1.43 (t, 3H, $J = 7.0$ Hz), 2.12-2.36 (m, 2H), 2.66 (t, 2H, $J = 6.8$ Hz), 3.10-3.36 (m, 2H), 4.44

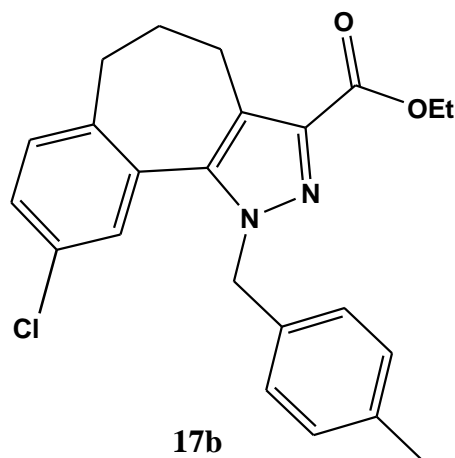
(q, 2H, $J = 7.2$ Hz), 6.65 (s, 1H), 7.12-7.28 (m, 2H), 7.35-7.48 (m, 2H), 7.57 (d, 1H, $J = 9.0$ Hz). Anal. ($C_{21}H_{17}Cl_3N_2O_2$) C, H, Cl, N.

Sintesi di Etil 8-cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazol-3-carbossilato (17a)



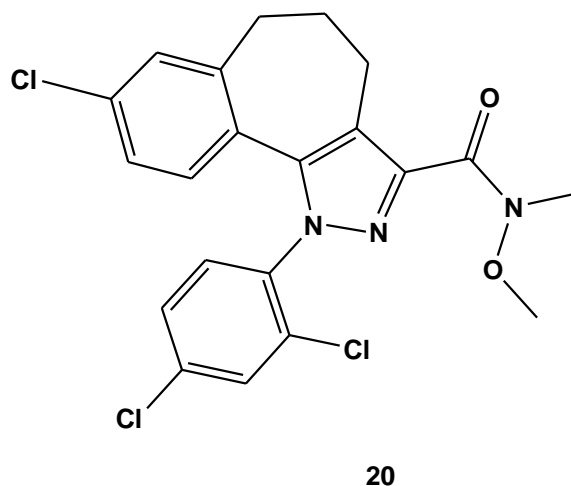
Viene utilizzata la procedura A2 per convertire 19a e 21 nel prodotto. Solido giallo; Resa 75%; R_f 0.42 (etere di petrolio/AcOEt, 8.5:1.5); IR 1725; 1H NMR ($CDCl_3$) δ 1.40 (t, 3H, $J = 7.1$ Hz), 2.18-2.36 (m, 2H), 2.36 (s, 3H), 2.66 (t, 2H, $J = 6.6$ Hz), 3.07-3.34 (m, 2H), 3.55 (s, 2H), 4.44 (q, 2H, $J = 7.1$ Hz), 6.61 (d, 1H, $J = 8.2$ Hz), 6.98 (dd, 1H, $J = 2.2$ and 8.1 Hz), 7.22 (d, 1H, $J = 2.1$ Hz), 7.22-7.30 (m, 4H). Anal. ($C_{23}H_{23}ClN_2O_2$) C, H, Cl, N.

Sintesi di Etil 9-cloro-1-(4'-metilbenzil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazol-3-carbossilato (17b)



Solido giallo; Resa 73%; R_f 0.40 (etere di petrolio/ AcOEt, 8.5:1.5); IR 1720; ^1H NMR (CDCl_3) δ : 1.42 (t, 3H, $J = 7.2$ Hz), 2.16-2.38 (m, 2H), 2.36 (s, 3H), 2.64 (t, 2H, $J = 6.7$ Hz), 3.05-3.32 (m, 2H), 3.56 (s, 2H), 4.45 (q, 2H, $J = 7.2$ Hz), 6.61 (d, 1H, $J = 8.0$ Hz), 7.00 (dd, 1H, $J = 2.0$ and 8.0 Hz), 7.24 (d, 1H, $J = 2.0$ Hz), 7.24-7.32 (m, 4H). Anal. ($\text{C}_{23}\text{H}_{23}\text{ClN}_2\text{O}_2$) C, H, Cl, N.

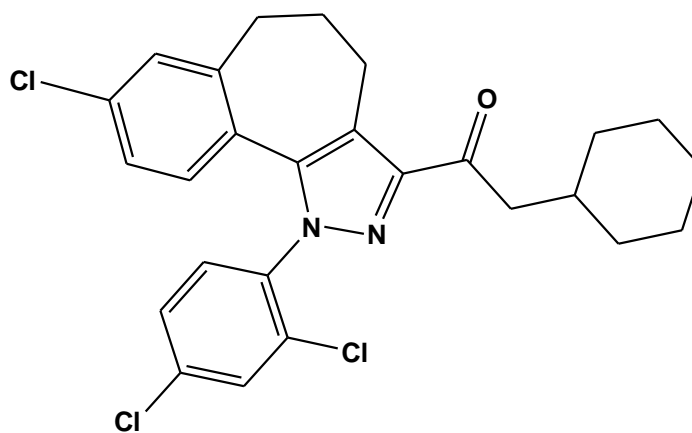
Preparazione di N-metossi-N-metil-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (20).



Trimetilaluminum (0.92 ml of 2 M soluzione in esano, 1.84 mmol) viene aggiunta goccia a goccia a una sospensione di dimetildrossilamina cloridrata (0.18 g, 1.84 mmol) in CH_2Cl_2 (3 ml) at 0°C . la soluzine viene lasciata a 0°C per 45 min e per 40 min a temperature ambiente. In questa soluzione, il composto 22a (0.4 g, 0.92 mmol) in CH_2Cl_2 (2 ml) viene aggiunto goccia goccia. La reazione

viene lasciata agire per 4 h a temperatura ambiente. La miscela viene raffreddata a 0°C, e aggiunto una soluzione al 10% HCl. La miscela viene estratta con CH₂Cl₂, lavata con acqua, ghiaccio, anidrificata su Na₂SO₄, e filtrato. Il residuo dopo evaporazione del solvente viene purificato mediante flash cromatografia (etere di petrolio/ AcOEt 7:3) ottenendo 0.33 g (80% resa) il composto **11** come solido bianco R_f 0.38 (etere di petrolio/ AcOEt 7:3); IR 1681; ¹H-NMR (CDCl₃) δ: 2.21-2.30 (m, 2H); 2.64-2.75 (m, 4H); 3.46 (s, 3H); 3.80 (s, 3H); 6.60 (d, 1H, J = 8.3 Hz); 7.02 (dd, 1H, J = 2.2 and 8.3 Hz); 7.30 (d, 1H, J = 1.6 Hz); 7.36 (dd, 1H, J = 2.2 and 8.3 Hz); 7.40 (d, 1H, J = 8.3 Hz); 7.45 (d, 1H, J = 2.2 Hz). Anal. calc. for C₂₁H₁₈Cl₃N₃O₂: C, 55.96; H, 4.03; Cl, 23.60; N, 9.32. Trovato: C, 55.82; H, 4.02; Cl, 23.57; N, 9.30.

Preparazione di 8-cloro-1-(2',4'-diclorofenil)-3-(1-ossocicloesil-1-il)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo (21).

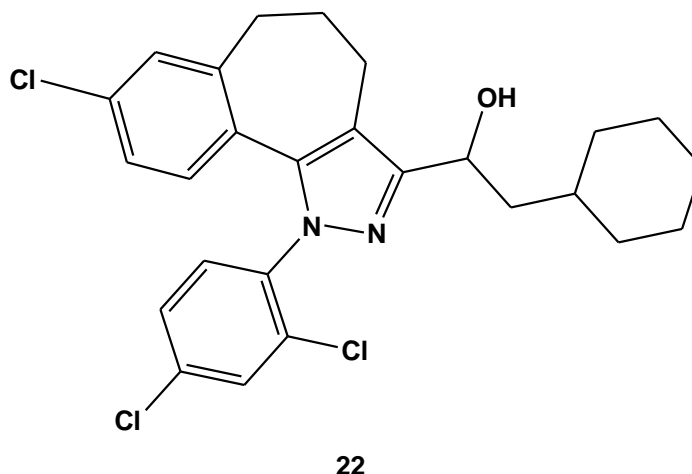


21

La soluzione di **26** (0.29 g, 0.64 mmol) in THF (6 ml) viene aggiunta goccia goccia a una soluzione di (cicloesilmetil)magnesio bromuro (3.86 ml of 0.5 M soluzione in THF, 1.93 mmol) a 0 °C sotto atmosfera di N₂. La miscela di reazione è stata gradualmente riscaldata a temperatura ambiente e agitata alla stessa temperatura per 24 h. Viene aggiunta goccia goccia una soluzione satura di NH₄Cl at 0 °C, la miscela di reazione viene diluita con EtOAc, e le due fasi separati. Lo strato acquoso è stato estratto con EtOAc e gli strati organici riuniti sono stati lavati con acqua, essiccati (Na₂SO₄), e filtrati. Il residuo dopo

evaporazione del solvente viene purificato da flash cromatografia (etere di petrolio/dietil etere 9:1) ottenendo 80 mg (26% resa) del composto **12** come un solido bianco. R_f 0.56 (etere di petrolio/dietil etere 9:1); IR 1685; $^1\text{H-NMR}$ (CDCl_3) δ 0.98-1.11 (m, 2H); 1.13-1.38 (m, 4H); 1.62-1.81 (m, 4H); 1.97-2.10 (m, 1H); 2.18-2.29 (m, 2H); 2.62-2.71 (m, 2H); 2.85-3.18 (m, 4H); 6.58 (d, 1H, $J = 8.3$ Hz); 7.00 (dd, 1H, $J = 2.2$ and 8.3 Hz); 7.30 (d, 1H, $J = 2.2$ Hz); 7.40 (dd, 1H, $J = 2.2$ and 8.3 Hz); 7.44-7.48 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ 25.3, 26.0, 26.2, 29.0, 32.9, 34.1, 37.4, 68.2, 121.7, 125.6, 125.7, 127.5, 127.6, 129.3, 129.4, 129.8, 129.9, 130.0, 132.0, 201.9. Anal. calc. for $\text{C}_{26}\text{H}_{25}\text{Cl}_3\text{N}_2\text{O}$: C, 64.01; H, 5.17; Cl, 21.80; N, 5.74. Trovato: C, 63.89; H, 5.16; Cl, 21.77; N, 5.72.

Preparazione di 8-cloro-1-(2',4'-diclorofenil)-3-(1-idrossi-2-cicloesilet-1-il)-1,4,5,6-tetraidrobenzo[6,7]ciclohepta[1,2-c]pirazolo (22).



Ad una sospensione di chetone 27 (60 mg, 0.12 mmol) in MeOH (3 ml) è stata aggiunta una soluzione di sodio boroidruro (10 mg, 0.25 mmol), e la miscela viene lasciata reagire a temperatura ambiente per 1.5 h. La miscela della reazione viene estratta con CHCl_3 e lavata con acqua. La fase organica è stata essiccata su sodio solfato anidro, filtrata, e concentrata per ottenere 60 mg (99% resa) del composto 28 come solido bianco. R_f 0.34 (etere di petrolio/acetato di etile 8:2); IR 3315; $^1\text{H-NMR}$ (CDCl_3) δ : 0.84-1.05 (m, 2H); 1.08-1.24 (m, 4H); 1.34-1.78 (m, 6H); 1.80-1.90 (m, 2H); 2.14-2.26 (m, 2H); 2.46-2.72 (m, 4H); 5.00 (bs, 1H); 6.60

(d, 1H, J = 8.3 Hz); 7.01 (dd, 1H, J = 2.2 and 8.3 Hz); 7.29 (d, 1H, J = 2.2 Hz); 7.35 (dd, 1H, J = 2.2 and 8.9 Hz); 7.40-7.45 (m, 2H); ^{13}C -NMR (CDCl_3) δ : 20.1, 26.2, 26.4, 26.6, 31.7, 32.5, 33.0, 34.1, 34.3, 45.5, 117.5, 126.2, 128.0, 128.1, 128.4, 129.8, 130.3, 130.5, 132.7, 133.7, 135.3, 136.5, 143.3, 154.3. Anal. calc. for $\text{C}_{26}\text{H}_{27}\text{Cl}_3\text{N}_2\text{O}$: C, 63.75; H, 5.56; Cl, 21.71; N, 5.72. Trovato: C, 63.68; H, 5.55; Cl, 21.69; N, 5.71.

Sintesi di N-piperidin-1-il-8-cloro-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepa[1,2-c]pirazolo-3-carbossamide (4a) e N-piperidin-1-il-1-(2',4'-diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepa[1,2-c]pirazolo-3-carbossamide (25c), procedura B.

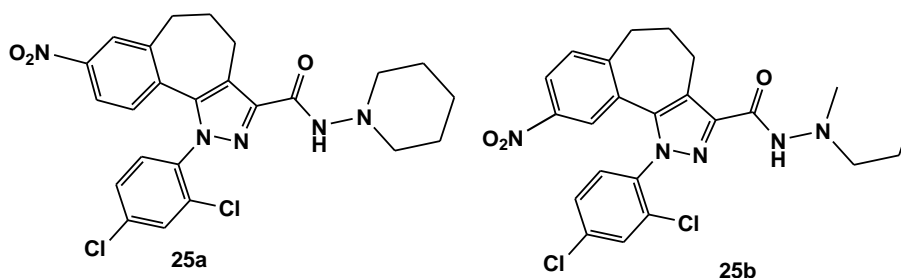
La polvere di ferro (1.30 g, 23.28 mmol) è stata sospesa in una miscela di acqua (15 ml) e etanolo (5.57 ml). HCl (0.82 ml) 1 N è stato aggiunto e la miscela è stata riscaldata fino a 50 °C. Dopo aggiunta di **25a** (1.08 g, 2.16 mmol) la miscela viene lasciata per 3 h a 65 °C. Dopo raffreddamento a RT viene aggiunto etil acetato, il ferro precipita e viene rimosso mediante filtrazione. La fase organica viene lavata con acqua, anidrificata su Na_2SO_4 , filtrato e concentrato. Il residuo purificato mediante flash cromatografia con CH_2Cl_2 /acetone 4/1 si ottiene così l'amina **32** (0.85 g, 84%) come solido giallo. Addizione di **26** (0.79 g, 1.68 mmol) è stata seguita da aggiunta a 0 °C di una soluzione di NaNO_2 (0.12 g, 1.74 mmol) in acqua (1 ml). Questa soluzione è stata agitata a 0 °C per 30 min e rapidamente aggiunta ad una soluzione raffreddata di CuCl (85 mg, 0.86 mmol) in HCl concentrato (2 ml). La miscela risultante è stata riscaldata a 55 °C per 1 h, raffreddata a RT, seguita da aggiunta di ghiaccio e acetato di etile. La fase organica è stata separata dalla fase acquosa. Le fasi organiche vengono lavate con acqua. Il solvente viene allontanato per evaporazione sotto vuoto. Il grezzo sottoposto a una purificazione cromatografica su gel di silice, utilizzando come eluente CH_2Cl_2 /acetone 9/1 viene ottenuto **4a** (0.22 g, 27%) e **25c** (50 mg, 6%) come solidi bianchi.

4a: mp 198-200°C, (202°C [12]). R_f 0.40 (dietilere/etere di petrolio, 6:4); IR (nujol): 3210, 1650; ^1H NMR (CDCl_3) δ 1.40-1.48 (m, 2H), 1.72-1.80 (m, 4H),

2.19-2.30 (m, 2H), 2.66 (t, 2H, $J = 6.6$ Hz), 2.83-2.90 (m, 4H), 2.90-3.48 (m, 2H), 6.56 (d, 1H, $J = 8.3$ Hz), 7.00 (dd, 1H, $J = 2.2$ and 8.3 Hz), 7.30 (d, 1H, $J = 2.2$ Hz), 7.40 (dd, 1H, $J = 2.2$ and 8.3 Hz); 7.42-7.48 (m, 2H), 7.66 (bs, 1H); ^{13}C NMR (CDCl_3) δ 20.2, 23.4, 25.5, 31.4, 32.6, 57.1, 122.7, 126.2, 127.7, 128.0, 128.2, 129.9, 130.4, 130.5, 132.5, 134.2, 135.9, 136.0, 142.2, 143.6, 143.8, 160.0. Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_3\text{N}_4\text{O}$ (C, H, Cl, N).

25c: mp 165-168 °C (167-169°C [18]). R_f 0.41 (CH_2Cl_2 / dietiletere 9/1); IR (nujol): 3165, 1650; ^1H NMR (CDCl_3) δ 1.39-1.48 (m, 2H), 1.70-1.79 (m, 4H), 2.21-2.31 (m, 2H), 2.68 (t, 2H, $J = 6.6$ Hz), 2.85-2.92 (m, 4H), 3.00-3.30 (m, 2H), 6.64 (bd, 1H, $J = 7.7$ Hz), 7.02 (td, 1H, $J = 1.3$ and 7.6 Hz), 7.21 (td, 1H, $J = 1.3$ and 7.5 Hz), 7.30 (bd, 1H, $J = 6.9$ Hz), 7.39 (dd, 1H, $J = 2.2$ and 8.4 Hz), 7.44 (d, 1H, $J = 2.2$ Hz), 7.46 (d, 1H, $J = 8.4$ Hz), 7.69 (bs, 1H). Anal. ($\text{C}_{24}\text{H}_{24}\text{Cl}_2\text{N}_4\text{O}$) C, H, Cl, N.

Sintesi di N-Piperidin-1-il-1-(2',4'-diclorofenil)-8-nitro-1,4,5,6-tetraidrobenzo [6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (25a) e N-Piperidin-1-il-1-(2',4'-diclorofenil)-9-nitro-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-c]pirazolo-3-carbossamide (25b)



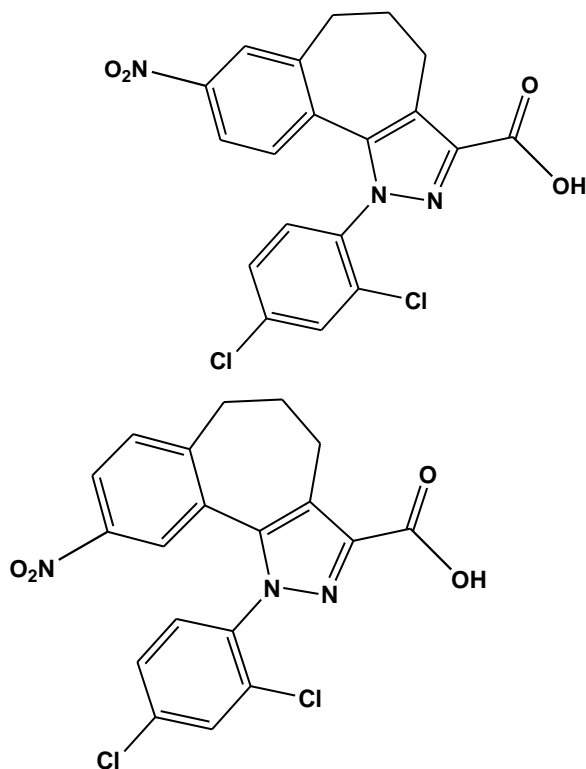
La miscela di 24a e 24b (2.80 g, 6.69 mmol) in acetonitrile (53 ml) viene aggiunta a una soluzione *N,N*-diisopropiletilamina (DIPEA) (2.56 ml, 14.72 mmol), *O*-(Benzotriazol-1-il)-*N,N,N',N'*-tetrametiluronioesafluorofosfato (HBTU) (3.04 g, 8.03 mmol) and 1-aminopiperidina (0.87 ml, 8.03 mmol) e viene lasciata reagire 1 notte a RT.. La miscela viene concentrata e il residuo lavato con etilacetato e acqua, anidrificato con Na_2SO_4 , filtrato e concentrato. Il residuo viene purificato mediante flash cromatografia utilizzando come eluenti dietiletere

per ottenere così la carbossamide 31a come solido bianco (Resa=33%) e **25b** (Resa=13%) come solido bianco.

25a: mp 178-180°C. R_f 0.77 (diethyl ether); ^1H NMR (CDCl_3) δ : 1.41-1.49 (m, 2H), 1.71-1.81 (m, 4H), 2.25-2.38 (m, 2H), 2.81 (t, 2H, $J = 6.6$ Hz), 2.83-2.92 (m, 4H), 3.20-3.60 (m, 2H), 6.80 (d, 1H, $J = 8.5$ Hz), 7.42-7.48 (m, 2H), 7.52 (d, 1H, $J = 9.0$ Hz), 7.67 (bs, 1H), 7.89 (dd, 1H, $J = 2.3$ and 8.5 Hz), 8.18 (d, 1H, $J = 2.3$ Hz). Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_3$ (C, H, Cl, N).

25b: mp 200-202°C. R_f 0.66 (dietiletere); ^1H NMR (CDCl_3) δ : 1.40-1.49 (m, 2H), 1.72-1.81 (m, 4H), 2.22-2.41 (m, 2H), 2.80 (t, 2H, $J = 6.8$ Hz), 2.84-2.90 (m, 4H), 3.19-3.58 (m, 2H), 7.43 (d, 1H, $J = 2.2$ Hz), 7.45-7.52 (m, 2H), 7.55 (d, 1H, $J = 2.3$ Hz), 7.59 (d, 1H, $J = 8.5$ Hz), 7.68 (bs, 1H), 8.06 (dd, 1H, $J = 2.3$ and 8.5 Hz). Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_3$ (C, H, Cl, N).

Sintesi di una miscela di 1-(2',4'-Diclorofenil)-8-nitro-1,4,5,6-tetraidrobenzo[6,7]ciclohepta[1,2-c]pirazol-3-carbossilic acid (**30a**) e 1-(2',4'-diclorofenil)-9-nitro-1,4,5,6-tetraidrobenzo[6,7]ciclohepta[1,2-c]pirazol-3-carbossilic acid (**24a** -**24b**)



Acido nitrico (1.5 mL) viene aggiunto lentamente a una soluzione di acido acetico (1.5 mL) raffreddato a 0 °C. 1-(2',4'-Diclorofenil)-1,4,5,6-tetraidrobenzo[6,7]cicloepita[1,2-*c*]pirazole-3-carbossilic acido (29) (0.50 g, 1.34 mmol) viene aggiunta alla miscela risultante e lasciata agire a RT per una notte. In seguito viene aggiunta una soluzione di ghiaccio e acetato di etile

Il precipitato formato viene raccolto per filtrazione, lavato con acqua ed essiccato a dare prodotto nitrazione grezzo (98 % di resa) come solido bianco che consiste del 30a 8-nitro e 30b 9-nitro regioisomeri in un rapporto molare di 5. R_f 0.38 (CHCl₃/MeOH 9:1); ¹H NMR (DMSO-d₆) (**24a**) δ 2.03-2.18 (m, 2H), 3.09-3.30 (m, 4H), 6.95 (d, 1H, J = 8.6 Hz), 7.67 (dd, 1H, J = 2.2 and 8.5 Hz), 7.81 (d, 1H, J = 2.2 Hz), 7.86 (d, 1H, J = 8.5 Hz), 7.97 (dd, 1H, J = 2.3 and 8.6 Hz), 8.29 (d, 1H, J = 2.2 Hz), 12.96 (bs, 1H). ¹H NMR (DMSO-d₆) (30b) δ 2.04-2.19 (m, 2H), 3.09-3.31 (m, 4H), 7.52 (d, 1H, J = 2.3 Hz), 7.68 (d, 1H, J = 8.5 Hz), 7.71 (dd, 1H, J = 2.2 and 8.6 Hz), 7.83 (d, 1H, J = 2.2 Hz), 7.92 (d, 1H, J = 8.5 Hz), 8.10 (dd, 1H, J = 2.4 and 8.4 Hz), 13.05 (bs, 1H).

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7 Articoli



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Pyrazoles with a “click” 4-[N-(4-fluorobutyl)-1,2,3-triazole] substituent in position 3 are nanomolar CB₁ receptor ligands

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ABSTRACT

Replacement of the 3-carboxylaminopiperidine substituent with a “click” 4-[N-(4-fluorobutyl)-(1,2,3-triazolyl)] group in Rimonabant-type pyrazoles produced a novel class of nanomolar CB₁ receptor ligands. Molecule 1d is the most promising lead with a K_i = 23 nM for CB₁, which is very close to that displayed by Rimonabant (SR141716), and fairly good CB₁/CB₂ selectivity (K_i CB₂/K_i CB₁ = 35.5), thus representing a promising candidate for [¹⁸F]radiolabeling and PET Imaging studies of the CB₁ receptor.

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1. Introduction

Cannabinoid receptors are members of the large family of G-protein coupled receptors (GPCRs) [1]. Two types of cannabinoid receptor have been discovered so far, CB₁ and CB₂ [2], and both of them have been extensively studied. CB₁ receptors are localised predominantly in the brain [2] whereas CB₂ receptors are more abundant in peripheral nervous system (PNS) cells [3], although some studies have shown the presence of CB₁ in the PNS [4] and of CB₂ in the central nervous system, albeit in low density [5]. CB₁ receptors have been associated with a number of disorders, including depression [6], anxiety [7], stress [8], schizophrenia [9], chronic pain [10] and obesity [11]. For this reason, several cannabinoid ligands were developed as drug candidates. Among these ligands, a prominent position is occupied by SR141716 (Rimonabant) [12], which is a pyrazole-core inverse agonist discovered by Sanofi-Synthelabo (now Sanofi-Aventis) in 1994, marketed in Europe as an anti-obesity drug but subsequently withdrawn from the market owing to its side-effects, which

included severe depression and suicidal thoughts. Since the relationship between (a) the CB₁ receptors' functional modification, density and distribution, and (b) the onset of a pathological state is still not well understood, the development of radio-ligands suitable for in vivo PET functional imaging of CB₁ receptors remains an important area of research in medicine and drug development. To date, a few radiotracers [13] based on the structure of SR141716 (Rimonabant) [12] have been synthesised and tested in vivo but most of them afforded unsatisfactory brain imaging results due to their poor ability to cross the blood-brain barrier (BBB). A handful of radiolabelled CB₁ PET ligands [14] have also been submitted to clinical trials in humans [15]. In this paper we describe the synthesis of a conceptually new class of high-affinity CB₁ ligands 1, bearing a “click” N-(4-fluorobutyl)-1,2,3-triazolyl function in position 3 of a pyrazolyl ring, as candidate PET tracers. Furthermore, we synthesised the 4-iodo-1,2,3-triazolyl analogue 10 which might be developed into a theranostic or a multi-modal imaging tool by radioiodination.

2. Results and discussion

2.1. Ligands design

Extensive theoretical and experimental structure-activity relationship studies have been performed on Rimonabant analogues for

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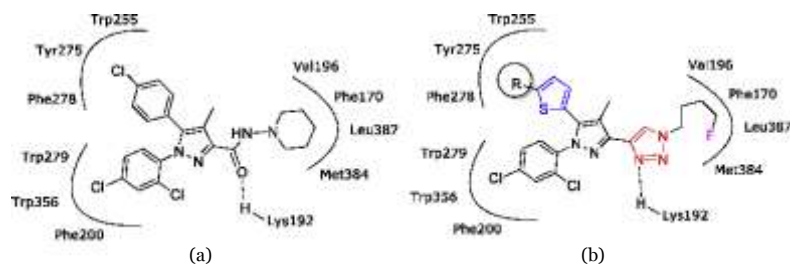


Fig. 1. (a) Rimonabant's pharmacophore. (b) Proposed binding mode of compounds 1 to CB1.

identifying a general pharmacophore. Hydrophobic interactions between ligands and CB1 receptor were deemed to be essential. In fact, the two aromatic rings in positions 1 and 5 of the pyrazole ring interact favourably with the residues Trp279/Phe200/Trp356 and Tyr275/Trp255/Phe278 respectively, and likewise the aminopiperidine cyclohexyl with the cavity constituted by Val196/Phe170/Leu387 and Met384 [16] (Fig. 1a). Moreover, the hydrogen bond between the ligand's amidic oxygen and the receptor residue Lys192 plays a crucial role in the binding, favouring the inverse agonism of Rimonabant.

With that in mind, we decided to replace the carbonyl-aminopiperidine residue in position 3 with a 4-(1,2,3-triazolyl) function, since either of the triazolyl sp^2 nitrogen atoms could act as hydrogen bond acceptor with Lys192. The 1,2,3-triazole would carry a *N*-(4-fluorobutyl) group, which should be readily amenable to [18 F]radiofluorination and could be accommodated in the lipophilic Val196/Phe170/Leu387/Met384 pocket. Finally, we planned to replace the 4-chlorophenyl group in 5-position with a 5-substituted 2-thiophenyl residue, which was previously shown to be a very advantageous structural modification leading to high-affinity CB1 ligands, such as NESS125A [17].

2.2. Synthesis of 1,2,3-triazolyl compounds 1

The synthesis of target compounds 1 envisaged the use of a key intermediate 8 (Scheme 1) which was obtained in a few synthetic steps from commercially available reagents such as diethyl oxalate, 1-(thiophen-2-yl) propan-1-one 2, and 2,4-dichlorophenylhydrazine hydrochloride, and directly converted

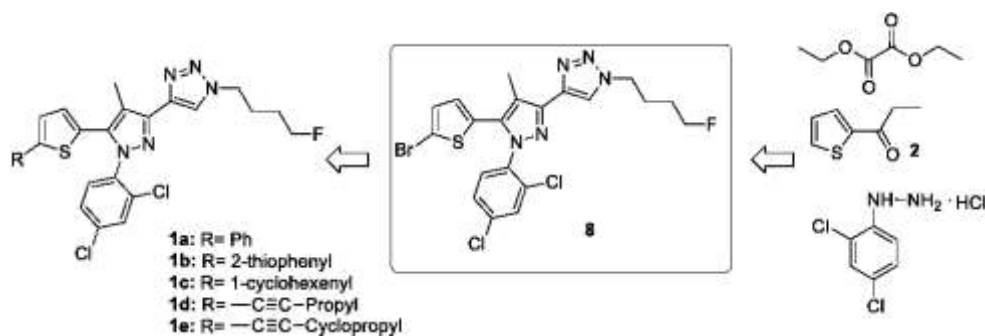
via a palladium-catalysed cross coupling reactions, into the desired target pyrazoles 1a–e.

The synthesis started from 2 (Scheme 2), which was condensed with diethyl oxalate in the presence of sodium ethylate to give, in 85% yield, the 1,3-diketoester 3 as a tautomeric mixture, predominantly containing the alkenylidene structure. Subsequently, tricarbonyl compound 3 and 2,4-dichlorophenylhydrazine were heated in ethanol [18] to afford the pyrazole 4 in rather modest yield (32%). The latter was regioselectively brominated, [19] employing NBS as bromine source, to afford the corresponding bromothiophene 5 in good yield (83%). The following conversion was accomplished through a DIBAL-H hydride reduction, providing the aldehyde 6 which was homologated under Bestmann-Ohira alkylation conditions [20] to generate the alkyne 7 in a moderate yield (55%). Finally, the key triazole 8 was achieved by means of a copper-catalysed azide-alkyne cycloaddition protocol [21] in an acceptable 55% yield.

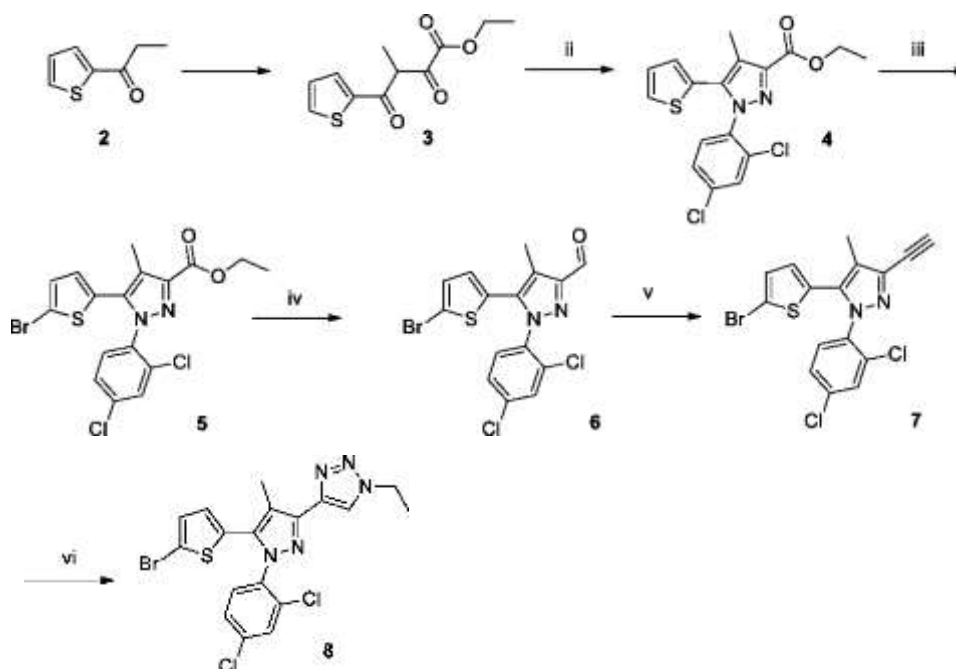
With the intermediate 8 in hand, compounds 1a–c were obtained by means of a palladium-catalysed Suzuki–Miyaura cross coupling [22] using the respective commercially available boronic acids, while compounds 1d–e were synthesised employing a copper-palladium catalysed Sonogashira cross coupling [23] using the appropriate alkyne (Scheme 3).

2.3. Synthesis of 5-iodo-1,2,3-triazolyl compound 10

The synthesis of 4-iodo-1,2,3-triazolyl derivative 10 (Scheme 4) started from the intermediate 7 that was iodinated in a good yield (74%) using 4-iodomorpholine as iodine source. Next, a



Scheme 1. Retro-synthesis of 1,2,3-triazolyl analogues 1a–e.



Scheme 2. Synthesis of key intermediate 8. Reagents and conditions: (i) diethyl oxalate, EtONa/EtOH, r.t., overnight, (85%); (ii) 2,4-dichlorophenylhydrazine hydrochloride, EtOH, reflux, overnight, (32%); (iii) NBS, CH₃CN, from 0 °C to r.t., overnight, (83%); (iv) DIBAL-H, DCM, -78 °C, 4 h, (55%); (v) dimethyl 1-diazo-2-oxopropylphosphonate, K₂CO₃, MeOH, r.t., overnight, (55%); and (vi) 1-azido-4-fluorobutane, CuI, sodium ascorbate, tert-BuOH/H₂O, r.t., overnight, (55%).

copper-catalysed azide-iodoalkyne cycloaddition [24] afforded the desired compound 10 in moderate yield (43%).

2.4. Biological tests

We next performed [³H]CP55940 displacement binding assays with membranes obtained from hCB₁ and hCB₂ CHO cells using methods we have described previously [25]. Results are summarised in Table 1 (affinity to CB₁ and CB₂ are expressed as K_i values).

Compound 1d stands out for its high CB₁ affinity, which was comparable to that displayed by Rimonabant (SR141716). Moreover, 1d showed fairly good CB₁/CB₂ selectivity (K_i CB₂/K_i CB₁ = 35.5). All the other compounds showed K_i CB₁ one order of magnitude higher than that of 1d, and low to moderate CB₁/CB₂ selectivity. Compound 1d is therefore the most promising candidate for further development, including its possible use as a PET tracer for imaging the CB₁ receptor in vivo.

Table 1

Compound	hCB ₁ CHO cells		hCB ₂ CHO cells	
	K _i ^a (95% CL ^b)	% Maximum displacement (95% CL ^b)	K _i ^a (95% CL ^b)	% Maximum displacement (95% CL ^b)
1a	200 (94.9–420)	76.5 (66.3–86.7)	2.36 × 10 ³ (641–8.70 × 10 ³)	73.3 (47.3–99.2)
1b	353 (103–1.20 × 10 ³)	85.1 (66.5–103)	1.70 × 10 ³ (953–3.02 × 10 ³)	97.5 (82.6–112)
1c	119 (40.2–353) ^f	80.0 (65.9–94.1)	471 (95.1–2.33 × 10 ³)	55.6 (41.8–69.5)
1d	23.4 (6.80–80.0)	75.6 (62.0–89.2)	830 (281–2.45 × 10 ³)	74.5 (55.0–94.0)
1e	164 (32.5–825)	58.8 (43.2–74.5)	n.a. ^c	n.a. ^c
8	312 (113–862)	100 (81.0–120)	1.02 × 10 ³ (603–1.72 × 10 ³)	92.0 (79.7–104)
10	422 (235–757)	71.8 (65.6–77.9)	n.a. ^c	n.a. ^c
SR141716	18.7 (11.1–31.4) ^e	90.2 (85.0–95.3)	1.40 × 10 ³ (500–3.70 × 10 ³) ^g	92.4 (70.4–114)

^a nM.

^b CL, confidence limits.

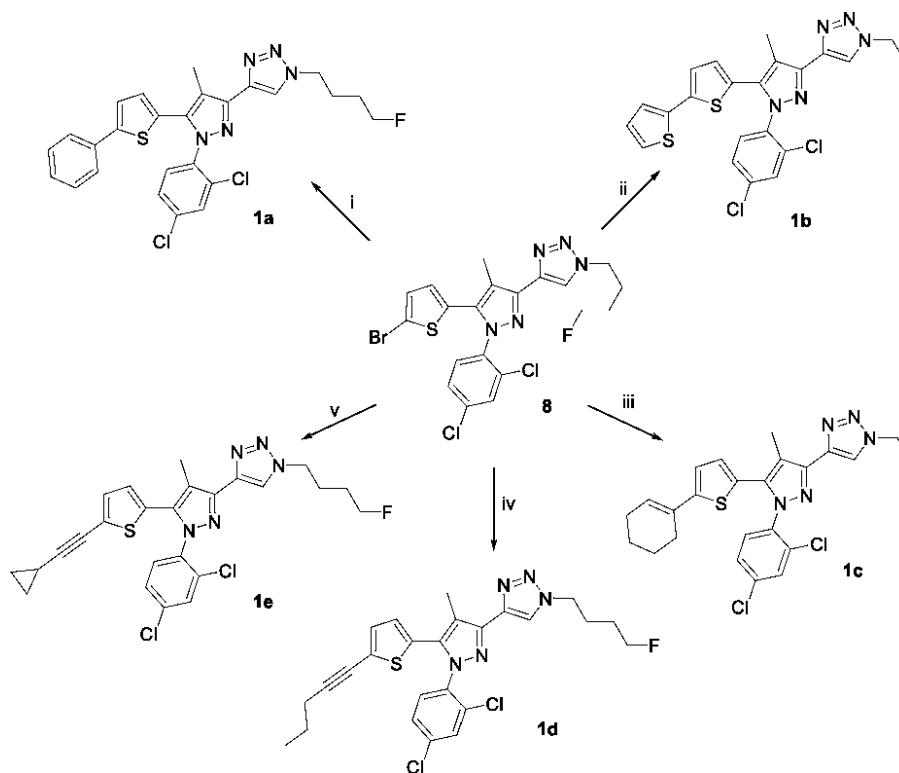
^c Plateau could not be reached.

^d n = 4, unless otherwise indicated.

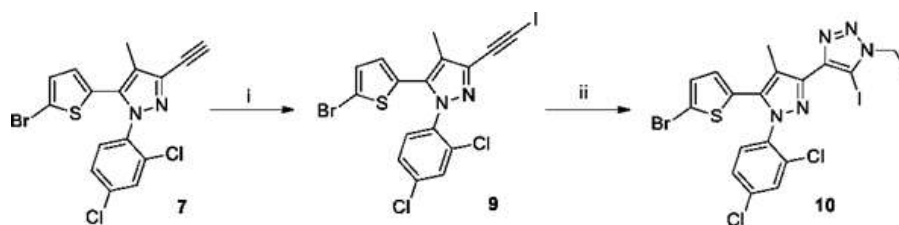
^e n = 14.

^f n = 12.

^g n = 2.



Scheme 3. Synthesis of analogues 7a–e. Reagents and conditions: (i) phenylboronic acid, Pd(PPh₃)₄, Na₂CO₃, DME, r.t., overnight, (35%); (ii) 2-thienylboronic acid, Pd(PPh₃)₄, Na₂CO₃, DME, r.t., overnight, (35%); (iii) 1-cyclohexen-1-yl-boronic acid, Pd(PPh₃)₄, Na₂CO₃, DME, r.t., overnight, (75%); (iv) 1-pentyne, Pd(PPh₃)₄, DIPEA, CuI, 80 °C, 20 h, (20%); and (v) cyclopropylacetylene, Pd(PPh₃)₄, DIPEA, CuI, 80 °C, 20 h, (25%).



Scheme 4. Synthesis of iodinated analogue 70. Reagents and conditions: (i) 4-iodomorpholine, CuI, THF, r.t. 1 h, (74%); and (ii) 1-azido-4-fluorobutane, CuI, TEA, THF, r.t., 72 h, (43%).

3. Conclusions

In Rimobant-type pyrazoles, replacement of the 3-carboxylaminopiperidine substituent with a “click” [4-(1,2,3-triazol-yl)] group carrying a N-(4-fluorobutyl) function produced a novel class of nanomolar CB₁ receptor ligands displaying nanomolar affinity for the CB₁ receptor. This may be explained by the capacity of the 1,2,3-triazole ring to mimic Rimobant’s 3-carboxamide residue and behave as hydrogen-bond acceptor with Lys192 of the CB₁ receptor. Molecule 7d is a particularly promising candidate for [¹⁸F]radiolabeling and PET Imaging studies of the CB₁ receptor, as it displayed a K_i = 23 nM for the CB₁,

in the same range as that displayed by Rimobant (SR141716), and fairly good CB₁/CB₂ selectivity (K_i CB₁ approx.36-fold lower than K_i CB₂).

4. Experimental

4.1. General information

¹H (400.13 MHz), ¹³C (100.58 MHz) and ¹⁹F (376.45 MHz) NMR spectra were recorded on a Bruker ADVANCE III spectrometer. ¹H NMR chemical shifts are reported relative to TMS, and the solvent

resonance was employed as the internal standard (CDCl_3 , $\delta = 7.26$). ^{13}C NMR spectra were recorded with complete proton decoupling, and the chemical shifts are reported relative to TMS with the solvent resonance as the internal standard (CDCl_3 , $\delta = 77.0$). ^{19}F NMR spectra were recorded with complete proton decoupling. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, m = multiplet, dd = doublet-doublet, dt = doublet-triplet, q = quartet. All chemical shifts (δ) are expressed in parts per million and coupling constant (J) are given in Hertz. LC–MS experiments were performed on an Agilent Technologies 1200 Series HPLC system equipped with a DAD and a 6120 MS detector composed by an ESI ionisation source and a Single Quadrupole mass selective detector using an Analytical C18 RP column (Phenomenex Luna, C18, 250 mm \times 4.60 mm, 5 μm , 100 $^\circ\text{A}$). HPLC purifications were performed on the Agilent 1200 system using a semi preparative C18 RP column (Phenomenex Luna, 250 mm \times 10.00 mm, 5 μm , 100 $^\circ\text{A}$). All reactions were carried out in oven- or flame-dried glassware under nitrogen atmosphere, unless stated otherwise. All commercially available reagents were used as received. Reactions were magnetically stirred and monitored by TLC on silica gel (60 F254 pre-coated glass plates, 0.25 mm thickness). Visualisation was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molybdate or KMnO_4 solution. Flash chromatography was performed on silica gel (60 $^\circ\text{A}$, particle size 0.040–0.062 mm). Yields refer to chromatographically and spectroscopically pure compounds, unless stated otherwise. Abbreviations used: DCM for dichloromethane, EtOAc for ethyl acetate, Et₂O for diethyl ether, NBS for *N*-bromosuccinimide, DIBAL-H for diisobutylaluminum hydride, DME for dimethoxyethane, DIPEA *N,N*-diisopropylethylamine, THF for tetrahydrofuran, MeOH for methanol and TEA for triethylamine. [^3H]CP55940 displacement binding assays with membranes obtained from hCB1 and hCB2 CHO cells using methods were performed as described previously [25].

4.2. Ethyl-3-methyl-2,4-dioxo-4-(thiophen-2-yl)butanoate (3)

Under a nitrogen atmosphere, sodium (0.86 g, 37.50 mmol) was added in small portions to dry ethanol (25 mL) and stirred at room temperature until all the sodium was dissolved. Diethyl oxalate (7.6 mL, 36.30 mmol) was then added, followed by dropwise addition of a solution of commercially available 1-(thiophen-2-yl)propan-1-one 2 (2.63 g, 18.65 mmol) in dry ethanol (26 mL). The mixture was stirred at room temperature for 18 h, then slowly poured into a mixture of ice and aqueous 1 N HCl. The resulting mixture was extracted with Et₂O, the organic layers were dried over MgSO_4 , filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 8:2) to give compound 3 (3.8 g, 85%) as a white solid. *R*_f 0.57 (Hexane/EtOAc 8:2); ^1H NMR (400 MHz, CDCl_3) δ : 1.24 (t, 3H, $J = 7.1$ Hz), 1.45 (d, 3H, $J = 7.0$ Hz), 4.25 (q, 2H, $J = 7.1$ Hz), 4.70 (q, 1H, $J = 7.0$ Hz), 7.37 (t, 1H, $J = 8.4$ Hz), 7.37 (d, 1H, $J = 8.4$ Hz), 7.51 (d, 1H, $J = 8.4$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 13.4, 14.0, 52.6, 63.1, 128.6, 133.5, 135.3, 142.4, 160.4, 187.5, 190.1; MS (ESI), calculated m/z $\text{C}_{11}\text{H}_{12}\text{O}_4\text{S}$: 241.0 $[\text{M}+\text{H}]^+$, 263.0 $[\text{M}+\text{Na}]^+$, found m/z (relative intensity): 241.0 $[\text{M}+\text{H}]^+$ (100), 263.0 $[\text{M}+\text{Na}]^+$ (40).

4.3. Ethyl 5-(thiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxylate (4)

The α,γ -diketoester 3 (4.63 g, 20.51 mmol) was dissolved in absolute EtOH (36 mL) and 2,4-dichlorophenylhydrazine hydrochloride (4.38 g, 20.51 mmol) was added in one portion, then the mixture was refluxed overnight. The solvent was removed under reduced pressure and the crude product was purified by flash

chromatography (Hexane/EtOAc 8:2). A final recrystallization (Hexane/EtOAc 7:3) gave compound 4 (2.01 g, 32%) as a white solid. *R*_f 0.30 (Hexane/EtOAc 8:2); ^1H NMR (400 MHz, CDCl_3) δ : 1.45 (t, 3H, $J = 7.1$ Hz), 2.46 (s, 3H), 4.47 (q, 2H, $J = 7.1$ Hz), 6.92 (dd, 1H, $J = 1.2$, 3.6 Hz), 7.02 (dd, 1H, $J = 3.6$, 5.1 Hz), 7.33 (dd, 1H, $J = 2.2$, 8.5 Hz), 7.38 (dd, 1H, $J = 1.2$, 5.1 Hz), 7.40 (d, 1H, $J = 8.5$ Hz), 7.46 (d, 1H, $J = 2.2$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 9.9, 14.5, 61.0, 120.0, 127.2, 127.7, 127.8, 128.6, 128.9, 130.0, 131.0, 133.9, 136.0, 136.3, 137.8, 142.9, 162.7; MS (ESI), calculated m/z $\text{C}_{17}\text{H}_{14}\text{Cl}_2\text{N}_2\text{O}_2\text{S}$: 381.0 $[\text{M}+\text{H}]^+$, 383.0 $[\text{M}+2+\text{H}]^+$, found m/z (relative intensity): 381.0 $[\text{M}+\text{H}]^+$ (100), 383.0 $[\text{M}+2+\text{H}]^+$ (70).

4.4. Ethyl 5-(5-bromothiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxylate (5)

Compound 4 (2.21 g, 1.31 mmol) was dissolved in acetonitrile (4.5 mL) and the solution was cooled to 0 $^\circ\text{C}$. NBS (0.39 g, 2.23 mmol) was added in small portions, then the mixture was stirred overnight at r.t. A saturated solution of $\text{Na}_2\text{S}_2\text{O}_3$ (5 mL) was added and the solvent was removed under reduced pressure. The resulting mixture was extracted with EtOAc, the organic layers were washed with water, brine, dried over MgSO_4 , filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 8:2) to give compound 5 (510 mg, 83%) as a pale yellow solid. *R*_f 0.38 (Hexane/EtOAc 8:2); ^1H NMR (400 MHz, CDCl_3) δ : 1.40 (t, 3H, $J = 7.1$ Hz), 2.41 (s, 3H), 4.43 (q, 2H, $J = 7.1$ Hz), 6.63 (d, 1H, $J = 3.9$ Hz), 6.94 (d, 1H, $J = 3.9$ Hz), 7.33 (d, 1H, $J = 2.0$ Hz), 7.35 (d, 1H, $J = 0.4$ Hz), 7.45 (dd, 1H, $J = 0.4$, 2.0 Hz); ^{13}C NMR (100 MHz, CDCl_3) δ : 10.1, 14.6, 61.2, 115.1, 120.5, 128.0, 129.4, 130.3, 130.3, 131.0, 133.9, 134.1, 135.8, 136.7, 137.0, 142.8, 162.6; MS (ESI), calculated m/z $\text{C}_{17}\text{H}_{13}\text{BrCl}_2\text{N}_2\text{O}_2\text{S}$: 458.9 $[\text{M}+\text{H}]^+$, 460.9 $[\text{M}+2+\text{H}]^+$, found m/z (relative intensity): 458.9 $[\text{M}+\text{H}]^+$ (65), 460.9 $[\text{M}+2+\text{H}]^+$ (100).

4.5. 5-(5-Bromothiophen-2-yl)-3-(2,4-dichlorophenyl)-5-methylcyclopenta-1,4-dienecarbaldehyde (6)

Ester 5 (0.2 g, 0.43 mmol) was dissolved in anhydrous dichloromethane (2 mL) and the mixture was cooled to -78 $^\circ\text{C}$. DIBAL-H (0.5 mL) was added drop wise over 45 min and the mixture was stirred for 4 h at -78 $^\circ\text{C}$. MeOH (0.5 mL) was added and the solvent was removed under reduced pressure. The resulting mixture was extracted with EtOAc, the organic layers were washed with brine, dried over MgSO_4 , filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 5:5) to give aldehyde 6 (0.45 g, 55%) as a white solid. *R*_f 0.50 (Hexane/EtOAc 5:5); ^1H NMR (400 MHz, CDCl_3) δ : 2.36 (s, 3H), 6.58 (d, 1H, $J = 3.9$ Hz), 6.90 (d, 1H, $J = 3.9$ Hz), 7.29 (d, 1H, $J = 0.5$ Hz), 7.30 (d, 1H, $J = 2.0$ Hz), 7.46 (dd, 1H, $J = 0.5$, 2.0 Hz), 10.0 (s, 1H); MS (ESI), calculated m/z $\text{C}_{15}\text{H}_9\text{BrCl}_2\text{N}_2\text{O}$: 414.9 $[\text{M}+\text{H}]^+$, 416.9 $[\text{M}+2+\text{H}]^+$, found m/z (relative intensity): 414.9 $[\text{M}+\text{H}]^+$ (55), 416.9 $[\text{M}+2+\text{H}]^+$ (100).

4.6. 2-Bromo-5-(5-(2,4-dichlorophenyl)-3-ethynyl-2-methylcyclopenta-1,3-dienyl) thiophene (7)

K_2CO_3 (0.11 g, 0.86 mmol) and dimethyl 1-diazo-2-oxopropylphosphonate (0.09 g, 0.52 mmol) were added to an ice cold solution of aldehyde 6 (0.45 g, 1.09 mmol) in MeOH (0.5 mL). After 5 min the ice bath was removed and the reaction was allowed to warm to r.t. and stirred for additional 12 h. Rochelle salt (2 mL) and Et₂O (2 mL) were added. The organic layers were washed with brine, dried over MgSO_4 , filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash

chromatography (Hexane/EtOAc 7:3) to give alkyne 7 (0.15 g, 55%) as a white solid. *R*_f 0.82 (Hexane/EtOAc 7:3); ¹H NMR (400 MHz, CDCl₃) S: 2.28 (s, 3H), 3.31 (s, 1H), 6.62 (d, 1H, *J* = 3.9 Hz), 6.96 (d, 1H, *J* = 3.9 Hz), 7.36 (d, 2H, *J* = 1.3 Hz), 7.50 (t, 1H, *J* = 1.3 Hz); ¹³C NMR (100 MHz, CDCl₃) S: 9.4, 75.0, 81.4, 114.5, 120.1, 127.9, 128.4, 130.1, 130.2, 130.7, 130.9, 133.7, 135.2, 135.8, 136.3, 136.4; MS (ESI), calculated *m/z* C₁₆H₉Br³⁵Cl₂N₂S: 410.9 [M+H]⁺, 412.9 [M+2+H]⁺, 432.9 [M+Na]⁺, 434.9 [M+2+Na]⁺, found *m/z* (relative intensity): 410.9 [M+H]⁺ (35), 412.9 [M+2+H]⁺ (70), 432.9 [M+Na]⁺ (55), 434.9 [M+2+Na]⁺ (100).

4.7. 1-Azido-4-fluorobutane

NaN₃ (0.25 g, 3.96 mmol) was added to a stirred solution of 1-bromo-4-fluorobutane (0.5 g, 2.61 mmol) in 40 mL of water/acetone (1:4). The resulting suspension was stirred at r.t. for 24 h. The mixture was extracted with DCM, dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. 1-Azido-4-fluorobutane was obtained (300 mg, 76%) as a yellow oil and was used without further purification. ¹H NMR (400 MHz, CDCl₃) S: 1.71–1.79 (m, 1H), 1.79–1.87 (m, 1H), 1.89–1.99 (m, 2H), 3.39 (t, 2H, *J* = 6.5 Hz), 4.42 (dt, 2H, *J*_{H-F} = 47.3 Hz, *J*_{H-H} = 5.7 Hz).

4.8. 4-(5-(5-Bromothiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl)-1-(4-fluorobenzyl)-1H-1,2,3-triazole (8)

Sodium ascorbate (0.08 g, 0.38 mmol) and copper sulphate (0.02 g, 0.07 mmol) were added to a solution of alkyne 7 (0.80 g, 1.94 mmol) and 1-azido-4-fluorobutane (0.34 g, 2.11 mmol) in *tert*-butanol/water (50 mL, 4:1). The mixture was stirred at r.t. for 24 h. A saturated aqueous solution of ammonium chloride (20 mL) was added and the aqueous layer was extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 6:4) to give triazole 8 (0.12 g, 55%) as a white solid. *R*_f 0.24 (Hexane/EtOAc 6:4); ¹H NMR (400 MHz, CDCl₃) S: 1.60–1.68 (m, 1H), 1.69–1.76 (m, 1H), 1.99–2.09 (m, 2H), 2.47 (s, 3H), 4.41 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 4.41 (t, 2H, *J* = 7.0 Hz), 6.60 (d, 1H, *J* = 3.9 Hz), 6.89 (d, 1H, *J* = 3.9 Hz), 7.25–7.32 (m, 2H), 7.42 (d, 1H, *J* = 2.1 Hz), 7.88 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) S: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) S: 10.3, 26.7 (d, *J*_{C-F} = 4.1 Hz), 26.7 (d, *J*_{C-F} = 20.1 Hz), 50.0, 83.3 (d, *J*_{C-F} = 165.5 Hz), 114.5, 121.3, 128.1, 128.9, 130.3, 130.4, 131.2, 134.1, 136.2, 136.3, 140.2, 142.4, 144.1, 147.0, 150.5; MS (ESI), calculated *m/z* C₂₀H₁₇Br₃₅Cl₂FN₅S: 528.0 [M+H]⁺, 530.0 [M+2+H]⁺, 550.0 [M+Na]⁺, 552.0 [M+2+Na]⁺, found *m/z* (relative intensity): 528.0 [M+H]⁺ (30), 530.0 [M+2+H]⁺ (65), 550.0 [M+Na]⁺ (52), 552.0 [M+2+Na]⁺ (100); HRMS calcd. for C₂₀H₁₈Br₃₅Cl₂FN₅S: 527.9822 and 529.9799, found: 527.9813 and 529.9787.

4.9. Suzuki–Miyaura cross coupling: general procedure

A mixture of alkyne 8 (0.18 mmol), Pd(PPh₃)₄ (0.09 mmol), the appropriate boronic acid (0.28 mmol) and aqueous Na₂CO₃ (0.23 mmol) in DME (2 mL), was heated to reflux and stirred overnight. The reaction was cooled down to r.t., poured into water, extracted with DCM, dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 6:4) to give the desired compound 1a–c.

4.10. 4-(5-(2,2'-bithiophen-5-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1a)

Starting from 8 and phenylboronic acid, compound 1a (46 mg, 35%) was obtained as yellow oil. *R*_f 0.36 (Hexane/EtOAc 6:4); ¹H

NMR (400 MHz, CDCl₃) S: 1.67–1.90 (m, 2H), 2.07–2.22 (m, 2H), 2.63 (s, 3H), 4.52 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 4.52 (t, 2H, *J* = 6.9 Hz), 6.88 (d, 1H, *J* = 3.8 Hz), 7.22 (d, 1H, *J* = 3.8 Hz), 7.30–7.46 (m, 6H), 7.51 (d, 1H, *J* = 2.2 Hz), 7.56 (d, 1H, *J* = 2.2 Hz), 7.98 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) S: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) S: 10.3, 26.5 (d, *J*_{C-F} = 4.1 Hz), 27.3 (d, *J*_{C-F} = 20.1 Hz), 49.8, 83.2 (d, *J*_{C-F} = 165.7 Hz), 121.2, 123.1, 125.8, 127.9, 128.0, 128.3, 129.0, 129.2, 129.7, 130.2, 130.4, 131.1, 133.6, 134.0, 135.9, 136.5, 136.8, 137.3, 142.5, 143.9, 146.2; MS (ESI), calculated *m/z* C₂₆H₂₂Cl₂FN₅S: 526.1 [M+H]⁺, 528.1 [M+2+H]⁺, found *m/z* (relative intensity): 526.1 [M+H]⁺ (100), 528.1 [M+2+H]⁺ (70); HRMS calcd. for C₂₆H₂₃Cl₂FN₅S: 526.1030, found: 526.1026.

4.11. 4-(1-(2,4-Dichlorophenyl)-4-methyl-5-(5-phenylthiophen-2-yl)-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1b)

Starting from 8 and 2-thienylboronic acid, compound 1b (48 mg, 35%) was obtained as yellow oil. *R*_f 0.31 (Hexane/EtOAc 6:4); ¹H NMR (400 MHz, CDCl₃) S: 1.71–1.79 (m, 1H), 1.79–1.86 (m, 1H), 2.10–2.20 (m, 2H), 2.62 (s, 3H), 4.52 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 4.52 (t, 2H, *J* = 7.0 Hz), 6.81 (d, 1H, *J* = 3.8 Hz), 7.04 (dd, 1H, *J* = 3.6, 5.1 Hz), 7.08 (d, 1H, *J* = 3.8 Hz), 7.17 (dd, 1H, *J* = 1.1, 3.6 Hz), 7.26 (dd, 1H, *J* = 1.1, 5.1 Hz), 7.38 (dd, 1H, *J* = 2.2, 8.4), 7.45 (d, 1H, *J* = 8.4 Hz), 7.52 (d, 1H, *J* = 2.2 Hz), 8.00 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) S: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) S: 10.2, 26.5 (d, *J*_{C-F} = 4.2 Hz), 27.3 (d, *J*_{C-F} = 20.1 Hz), 49.8, 83.2 (d, *J*_{C-F} = 165.7 Hz), 115.5, 120.7, 123.6, 124.2, 125.0, 127.9 (2C), 129.0, 130.2, 131.1, 134.0, 136.0, 136.3, 136.5, 137.1, 139.3, 142.4, 143.9, 146.1; MS (ESI), calculated *m/z* C₂₄H₂₀35Cl₂FN₅S₂: 532.1 [M+H]⁺, 534.1 [M+2+H]⁺, found *m/z* (relative intensity): 532.1 [M+H]⁺ (100), 534.1 [M+2+H]⁺ (75); HRMS calcd. for C₂₄H₂₁Cl₂FN₅S₂: 532.0594, found: 532.0588.

4.12. 4-(5-(5-Cyclohexenylthiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1c)

Starting from 8 and 1-cyclohexen-1-yl-boronic acid, compound 1c (15 mg, 75%) was obtained as yellow oil. *R*_f 0.36 (Hexane/EtOAc 6:4); ¹H NMR (400 MHz, CDCl₃) S: 1.58–1.71 (m, 4H), 1.71–1.84 (m, 4H), 2.16–2.23 (m, 2H), 2.34–2.40 (m, 2H), 2.58 (s, 3H), 4.51 (t, 2H, *J* = 7.0 Hz), 4.51 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 6.15–6.18 (m, 1H), 6.73 (d, 1H, *J* = 3.8 Hz), 6.82 (d, 1H, *J* = 3.8 Hz), 7.34 (dd, 1H, *J* = 2.2, 8.5 Hz), 7.40 (d, 1H, *J* = 8.5 Hz), 7.50 (d, 1H, *J* = 2.2 Hz), 7.98 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) S: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) S: 10.2, 22.0, 22.6, 25.6, 26.5 (d, *J*_{C-F} = 4.0 Hz), 27.4 (d, *J*_{C-F} = 22.0 Hz), 29.7, 49.8, 83.2 (d, *J*_{C-F} = 165.7 Hz), 115.2, 121.1, 125.2, 126.3, 127.8, 128.1, 128.5, 130.1, 130.3, 130.7, 131.1, 134.0, 135.7, 136.6, 142.0, 143.8, 149.0; MS (ESI), calculated *m/z* C₂₆H₂₆35Cl₂FN₅S: 530.1 [M+H]⁺, 532.1 [M+2+H]⁺, found *m/z* (relative intensity): 530.1 [M+H]⁺ (100), 532.1 [M+2+H]⁺ (70); HRMS calcd. for C₂₆H₂₇Cl₂FN₅S: 530.1333, found: 530.1343.

4.13. Sonogashira reaction: general procedure

A mixture of alkyne 8 (0.09 mmol), Pd(PPh₃)₄ (0.003 mmol), DIPEA (1 mL) and the appropriate alkyne (0.19 mmol of either 1-pentyne or cyclopropylacetylene) was stirred at 40 °C for 20 min. CuI (0.006 mmol) was added and the reaction was stirred at 80 °C overnight. The mixture was cooled down to r.t., diluted with EtOAc (1 mL), dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified first by HPLC (Semi-preparative C18 Luna column, Eluent: A: H₂O, Eluent B: CH₃CN; in isocratic condition Eluent A: 20% and Eluent B: 80%, 5 mL/min) to give the desired compound 1d–e.

4.14. 4-(1-(2,4-Dichlorophenyl)-4-methyl-5-(5-(pent-1-ynyl)thiophen-2-yl)-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1d)

Starting from 8 and 1-pentyne, compound 1d (50 mg, 20%) was obtained as a yellow oil after an HPLC purification (Semi-preparative C18 Luna column, Eluent A: H₂O, Eluent B: CH₃CN; in isocratic condition Eluent A: 20% and Eluent B: 80%, 5 mL/min, retention time: 8.9 min). R_f 0.24 (Hexane/EtOAc 6:4); ¹H NMR (400 MHz, CDCl₃) δ: 1.02 (t, 3H, *J* = 7.4 Hz), 1.52–1.66 (m, 2H), 1.68–1.75 (m, 1H), 1.75–1.85 (m, 1H), 2.04–2.19 (m, 2H), 2.39 (t, 2H, *J* = 7.1 Hz), 2.55 (s, 3H), 4.48 (t, 2H, *J* = 7.0 Hz), 4.49 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 6.77 (d, 1H, *J* = 3.8 Hz), 6.99 (d, 1H, *J* = 3.8 Hz), 7.33 (dd, 1H, *J* = 2.1, 8.4 Hz), 7.38 (d, 1H, *J* = 8.4 Hz), 7.48 (d, 1H, *J* = 2.1 Hz), 7.96 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) δ: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: 10.3, 13.7, 21.8, 22.1, 26.5 (d, *J*_{C-F} = 4.2 Hz), 27.3 (d, *J*_{C-F} = 20.1 Hz), 29.8, 50.0, 73.3, 83.3 (d, *J*_{C-F} = 165.7 Hz), 96.6, 115.6, 116.5, 121.3, 126.4, 128.1, 130.4, 131.1, 131.2, 134.1, 136.1, 136.3, 137.0, 142.4, 144.0; MS (ESI), calculated *m/z* C₂₅H₂₄Cl₂N₃: 516.1 [M+H]⁺, 518.1 [M+2+H]⁺, found *m/z* (relative intensity): 516.1 [M+H]⁺ (100), 518.1 [M+2+H]⁺ (75); HRMS calcd. for C₂₅H₂₄Cl₂N₃: 516.1186, found: 516.1176.

4.15. 4-(5-(5-(2-cyclopropylethynyl)thiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl)-1-(4-fluorobutyl)-1H-1,2,3-triazole (1e)

Starting from 8 and cyclopropylacetylene, compound 1e (40 mg, 25%) was obtained as a yellow oil after an HPLC purification (Semi-preparative C18 Luna column, Eluent: A: H₂O, Eluent B: CH₃CN; in isocratic condition Eluent A: 20% and Eluent B: 80%, 5 mL/min, Retention time: 12.0 min). R_f 0.24 (Hexane/EtOAc 6:4); ¹H NMR (400 MHz, CDCl₃) δ: 0.77–0.86 (m, 2H), 0.87–0.94 (m, 2H), 1.41–1.54 (m, 1H), 1.70–1.77 (m, 1H), 1.77–1.85 (m, 1H), 2.08–2.19 (m, 2H), 2.56 (s, 3H), 4.51 (t, 2H, *J* = 7.0 Hz), 4.51 (dt, 2H, *J*_{H-F} = 47.2 Hz, *J*_{H-H} = 5.7 Hz), 6.69 (d, 1H, *J* = 3.9 Hz), 6.98 (d, 1H, *J* = 3.9 Hz), 7.33–7.41 (m, 2H), 7.51 (d, 1H, *J* = 2.0 Hz), 7.98 (s, 1H); ¹⁹F NMR (376.45 MHz, CDCl₃) δ: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: -1.47, 8.50, 8.68 (2C), 26.5 (d, *J*_{C-F} = 4.1 Hz), 27.0 (d, *J*_{C-F} = 19.2 Hz), 46.1, 49.8, 68.0, 83.2 (d, *J*_{C-F} = 165.5 Hz), 90.2, 115.6, 118.7, 121.1, 126.1, 126.7, 127.9, 128.5, 129.5, 130.1, 131.2, 133.9, 135.9, 137.6, 143.4; MS (ESI), calculated *m/z* C₂₅H₂₂Cl₂N₃: 514.1 [M+H]⁺, 516.1 [M+2+H]⁺, found *m/z* (relative intensity): 514.1 [M+H]⁺ (100), 516.1 [M+2+H]⁺ (75); HRMS calcd. for C₂₅H₂₂Cl₂N₃: 514.1030, found: 514.1020.

4.16. 4-Iodomorpholine

To a solution of iodine (4 g, 31.5 mmol) in MeOH (63 mL) morpholine (2.75 mL, 31.5 mmol) was added drop wise and the mixture was stirred for 1 h. The precipitate formed was collected by filtration, dried under vacuum and used crude, without further purification. R_f 0.32 (DCM/MeOH 95:5); ¹H NMR (400 MHz, CDCl₃) δ: 2.89–3.00 (m, 2H), 3.69–3.77 (m, 2H).

4.17. 5-(5-Bromothiophen-2-yl)-1-(2,4-dichlorophenyl)-3-(iodoethynyl)-4-methyl-1H-pyrazole (9)

CuI (20.0 mg, 0.07 mmol) and 4-iodomorpholine (340 mg, 1.56 mmol) were added to a solution of alkyne 7 (590 mg, 1.43 mmol) in THF (4 mL). The reaction mixture was stirred at room temperature for 1 h. The mixture was filtered on a neutral alumina pad and the solvent was evaporated under reduced pressure. The iodinated derivative 9 was obtained (570 mg, 74%) as a yellow oil and was used without further purification. R_f 0.84 (Hexane/EtOAc 7:3); ¹H NMR (400 MHz, CDCl₃) δ: 2.16 (s, 3H), 6.51

(d, 1H, *J* = 3.9 Hz), 6.83 (d, 1H, *J* = 3.9 Hz), 7.25 (m, 2H), 7.38 (m, 1H); MS (ESI), calculated *m/z* C₁₆H₈Br³⁵Cl₂N₂S: 536.8 [M+H]⁺, 538.8 [M+2+H]⁺, 558.8 [M+Na]⁺, 560.8 [M+2+Na]⁺, found *m/z* (relative intensity): 536.8 [M+H]⁺ (20), 538.8 [M+2+H]⁺ (50), 558.8 [M+Na]⁺ (70), 560.8 [M+2+Na]⁺ (100).

4.18. 4-[5-(5-Bromothiophen-2-yl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-yl]-1-(4-fluorobutyl)-5-iodo-1H-1,2,3-triazole (10)

1-Azido-4-fluorobutane (400 mg, 0.37 mmol), CuI (40 mg, 0.19 mmol) and TEA (10 mL, 0.74 mmol) were added to a solution of propargyl iodide 9 (200 mg, 0.37 mmol) in THF (5 mL). The reaction was stirred at r.t. for 72 h, then quenched with a 10% aqueous solution of NH₄OH (10 mL) and the solvent was removed under reduced pressure. The residue was diluted with Et₂O, washed with water, dried over MgSO₄, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography (Hexane/EtOAc 4:1) to give the triazole 10 (100 mg, 42%) as a white solid. R_f 0.52 (Hexane/EtOAc 4:1); ¹H NMR (400 MHz, CDCl₃) δ: 1.70–1.79 (m, 1H), 1.79–1.87 (m, 1H), 2.05–2.19 (m, 2H), 2.41 (s, 3H), 4.50 (dt, 2H, *J*_{H-H} = 5.8 Hz, *J*_{C-F} = 47.2 Hz), 4.54 (t, 2H, *J* = 7.1 Hz), 6.66 (d, 1H, *J* = 3.9 Hz), 6.96 (d, 1H, *J* = 3.9 Hz), 7.30–7.38 (m, 2H), 7.50 (d, 1H, *J* = 1.9 Hz); ¹⁹F NMR (376.45 MHz, CDCl₃) δ: -219.3 (s, 1F); ¹³C NMR (100 MHz, CDCl₃) δ: 10.1, 26.0 (d, *J*_{C-F} = 4.4 Hz), 27.3 (d, *J*_{C-F} = 20.3 Hz), 50.3, 78.0, 83.1 (d, *J*_{C-F} = 165.8 Hz), 114.4, 116.9, 127.9, 128.7, 130.1, 130.3, 130.9, 131.2, 133.9, 135.9, 136.0, 136.2, 143.1, 145.1; MS (ESI), calculated *m/z* C₂₀H₁₆Br₃₅Cl₂N₃S: 653.9 [M+H]⁺, 655.9 [M+2+H]⁺, found *m/z* (relative intensity): 653.9 [M+H]⁺ (75), 655.9 [M+2+H]⁺ (100); HRMS calcd. for C₂₀H₁₆Br₃₅Cl₂N₃S: 653.8788 and 655.8765, found: 653.8784 and 655.8757.

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A critical review of both the synthesis approach and the receptor profile of the 8-chloro-1-(2',4'-dichlorophenyl)-N-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide and analogue derivatives.

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Abstract

8-Chloro-1-(2',4'-dichlorophenyl)-N-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide **9a** was discovered as potent and selective CB₁ antagonist by part of our group few years ago. In particular it was reported to have an affinity towards the CB₁ cannabinoid receptor (CB1), expressed as K_i of 0.00035 nM. Nevertheless significantly divergent data were reported for the same compound from other laboratories. To define unequivocally the receptor profile of **9a**, we have critically reviewed both its synthesis approach and binding data. Here we report that, in contrast to our previously reported data, **9a** showed a K_i value for CB1 in the order of nanomolar rather than of femtomolar range. The novel determined receptor profile of **9a** was also ascertained for analogue derivatives **9b-i**.

Although the cannabinoid receptor profile of **9a** was reviewed in this work, the relevance of this compound in CB1 antagonist based drug discovery is confirmed.

Keywords: Cannabinoids; endocannabinoid system (ECS); 8-chloro-1-(2',4'-dichlorophenyl)-*N*-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide; cannabinoid receptor binding assays; CB₁ receptor ligands.

1. Introduction

The endocannabinoid system (ECS) consists of CB₁ and CB₂ cannabinoid receptors (CB₁ and CB₂R, respectively), endogenous ligands such as anandamide (*N*-arachidonylethanolamine, AN) and 2-arachidonoylglycerol (2-AG), and several proteins responsible for their synthesis and degradation [1-3]. The physiological role of cannabinoid receptors seems to be related to certain pathophysiological processes such as appetite modulation, pain, immune system modulation [4]. In particular, selective CB₁ agents might produce beneficial therapeutic effects for the prevention and/or the treatment of various disorders and diseases as anorexia, emesis, pain, neurodegenerative diseases, glaucoma, inflammation, sexual dysfunctions, multiple sclerosis, metabolic syndrome, obesity, and immune system disorders [5-9]. CB₁ antagonists have been proposed as drugs for the prevention of weight gain and the treatment of obesity with improvement of cardiovascular and metabolic risk factors, even if the first candidate within this class, Acomplia[®] (rimonabant, Figure 1) by Sanofi Aventis [10], has been withdrawn from European market due to its adverse effects [11].

In previous communications [12,13], the synthesis and structure-affinity relationships (SAR) regarding CB₁ and CB₂R of 1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazoles of general structure **I** (Figure 1) were described. These compounds were also reported as general classes in a patent application by Sanofi-Synthélabo (now Sanofi Aventis) [14], and partially reviewed by Lange *et al* and Padgett [15]. Moreover, further SAR studies were reported on various series of condensed tricyclic pyrazole based cannabinoids (series **II-VI**) by evidencing key structural parameters in the modulation of CB₁ rather than CB₂ affinity and selectivity (Figure 2) [16].

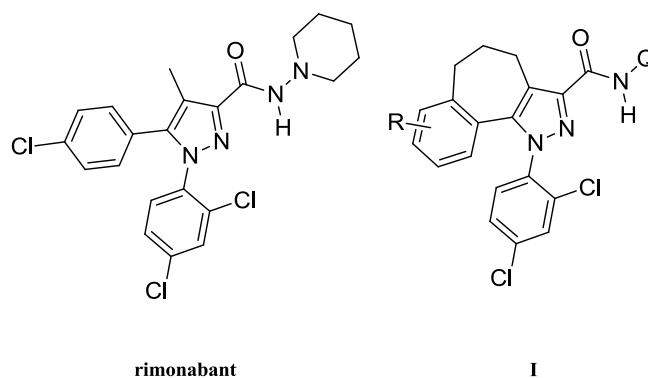


Fig. 1. Rimonabant and corresponding rigid analogues **I**.

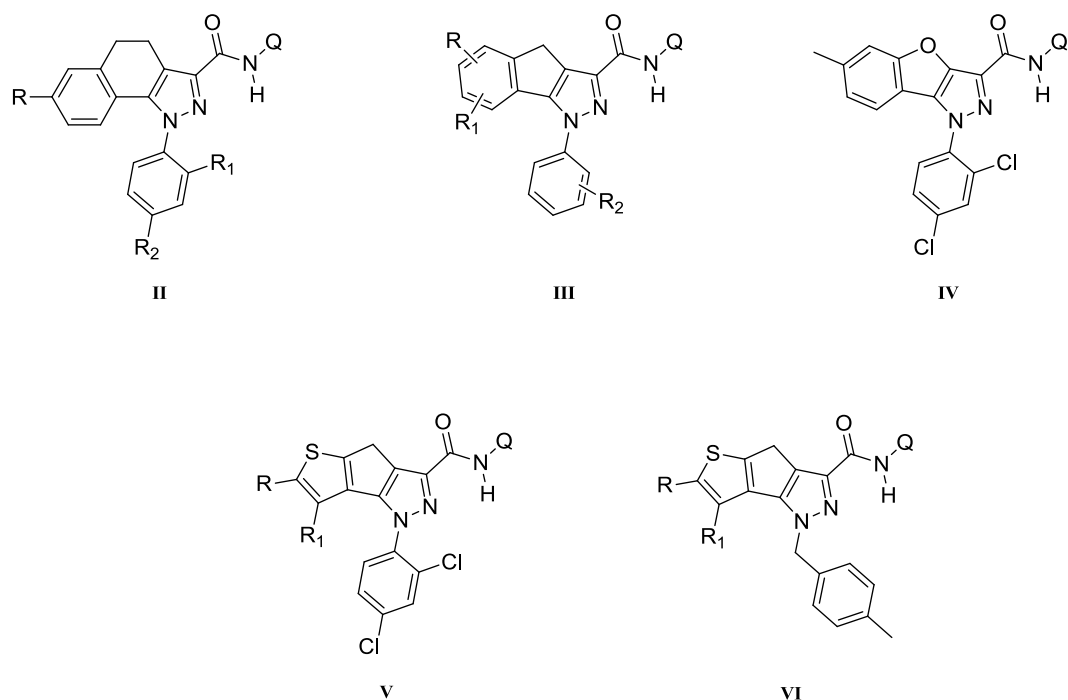
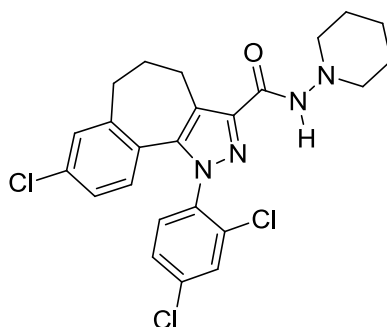


Fig. 2. Series of conformationally constrained tricyclic pyrazole based cannabinoid ligands having significant activity to CB₁ and/or CB₂ receptors [16].

In particular it was reported by part of our group that one compound belongs to series **I**, the 8-chloro-1-(2',4'-dichlorophenyl)-N-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide, namely **NESS0327** (Fig. 3), showed remarkable binding at the CB₁ and selectivity vs the CB₂R. In fact, $K_i\text{CB}_1 = 0.00035 \pm 0.000005$ nM and $K_i\text{CB}_2 = 21 \pm 0.5$ nM were previously reported as affinity values of **NESS0327** as determined using [³H]CP-55,940 and mouse brain membrane and mouse spleen homogenates, respectively [12]. The compound behaved as a CB₁ antagonist in both *in vivo* tests (mouse WIN 55,212-2 induced antinociception) and *in ex vivo* assays (isolated organs: mouse vas deferens) [12]. Moreover CB₁ neutral antagonism instead of inverse agonism of lead compound rimonabant was highlighted for **NESS0327** according to [³⁵S]GTPγS binding assay results [12].



NESS0327

Fig. 3. Structure of **NESS0327** [12].

Independently, two other groups [17-18] described the synthesis and biological evaluation of conformationally constrained analogues of rimonabant. Amongst these cannabinoid derivatives, they prepared one compound with the same structure of **NESS0327** which showed an affinity for the CB1 markedly different from that previously reported by our group. Values of K_i CB₁ equal to 126 nM and 18.4 nM were in fact determined, using [³H]CP-55,940 and hCB₁ receptor cloned in Chinese hamster ovary (CHO) cells, or membranes isolated from a HEK-293 expression system, respectively [18]. The compound was synthesized both according to the same procedure adopted by our group [12] and through a different protocol [17].

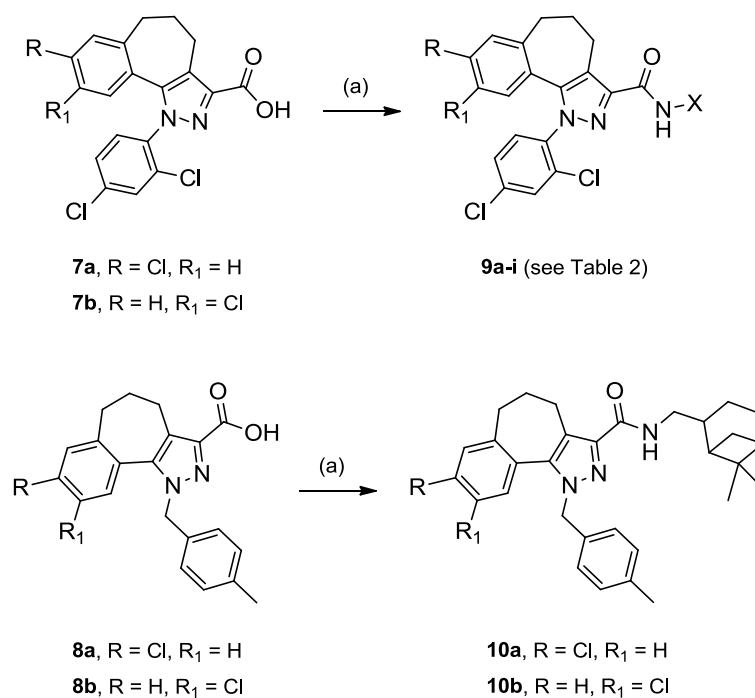
To further investigate the discrepancy between these binding data, we re-synthesized the tricyclic derivative 8-chloro-1-(2',4'-dichlorophenyl)-*N*-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7] cyclohepta[1,2-*c*]pyrazole-3-carboxamide. Two different methods were adopted: the synthetic route previously developed by us (Schemes 1 and 2) [12-13], namely "Procedure A", based on the obtainment of the chlorine-substituted 1,3-diketoester **2a** as intermediates, and the modification reported by Stoit *et al.* (Scheme 4) [17], namely "Procedure B". The last was characterized by the synthesis of the corresponding nitro-compound of **NESS0327**, and the following steps involving the nitro group reduction and the amino group replacement by the chlorine atom. The two final products were then compared both by chemical analysis and by cannabinoid receptor binding assays.

Moreover, structure relationship activity (SAR) study was performed by acting on the substituents at both the position 1 and 3 of the pyrazole ring of the 1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole scaffold. General Procedure A was adopted to give the series of the investigated analogues of **NESS0327**. In vitro assays were performed to preliminary investigate intrinsic activity of lead compounds selected according to the determined binding profile.

2. Chemistry

2.1. Procedure A: Synthesis of NESS0327 and analogue carboxamides (**9a-i**, **10a**, and **10b**)

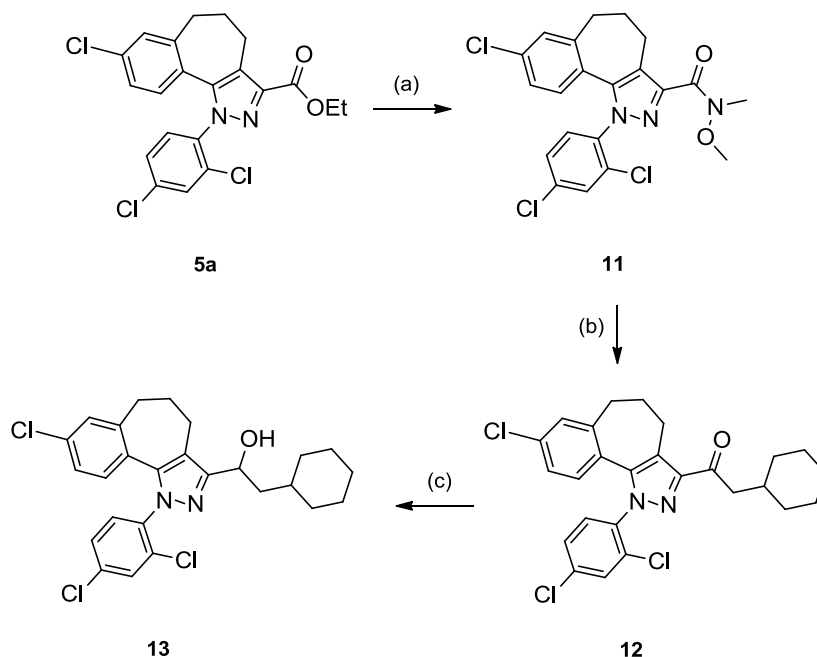
As shown in Scheme 2, the acids **7a-b** were allowed to react with the required amines or hydrazines in the presence of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole hydrate (HOBt) to give the desired compounds **9a-i**. Similarly, carboxamides **10a-b** were obtained starting from **8a-b** (Scheme 2).



Scheme 2. Reagents and conditions: (a) EDC, HOBt, X-NH₂, CH₂Cl₂.

2.2. Synthesis of derivatives **11** and **12**.

Weinreb amide **11** was synthesized from the ester **5a** by reaction with *N,O*-dimethylhydroxylamine hydrochloride in the presence of trimethylaluminum (Scheme 3). Reaction of **11** with (cyclohexylmethyl)magnesium bromide in anhydrous tetrahydrofuran tricyclic ketone **12**. Finally, the carbonyl group of **12** was reduced by sodium borohydride at room temperature to obtain the racemic alcohol (±)-**13**.

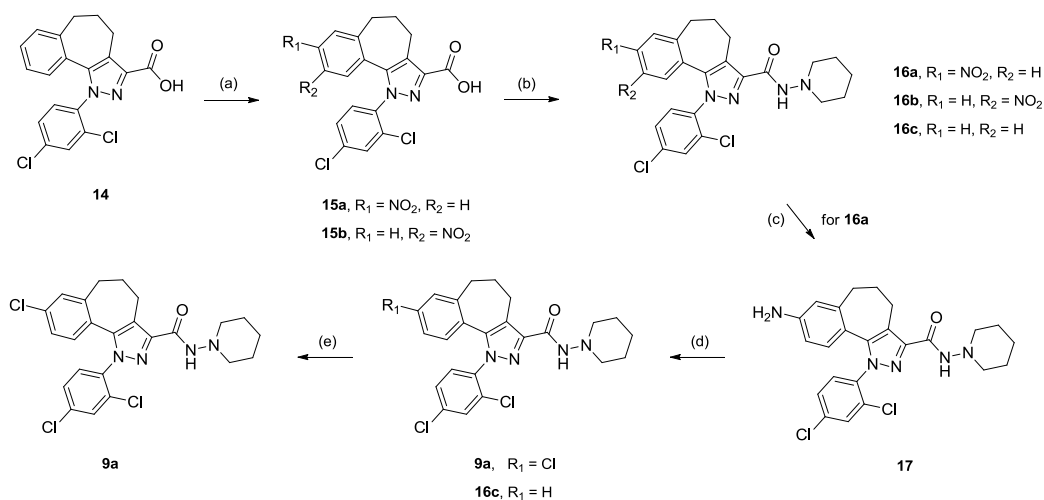


Scheme 3. Reagents and conditions: (a) AlMe_3 , $\text{MeNHOMe} \cdot \text{HCl}$, CH_2Cl_2 ; (b) (cyclohexylmethyl)magnesium bromide, THF; (c) NaBH_4 , MeOH.

2.3 Procedure B: Synthesis of NESS0327

As shown in Scheme 4, the second route to obtain **9a** was defined according to the previously reported procedure developed by Stoit *et al.* (Scheme 4) [17]. Nitration of the tricyclic acid 1-(2',4'-dichlorohenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid **14** [13], achieved by fuming nitric acid in acetic acid, gave a mixture of the 8- and 9-nitro-isomers **15a** and **15b**, respectively, in nearly quantitative yield. This mixture of regioisomers was reacted with 1-aminopiperidine in the presence of *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA), followed by chromatographic separation of the resulting regioisomers, to give the 8-nitrohydrazide **16a** and the 9-nitrohydrazide **16b** in 46% overall yield, as well as a residue of the unsubstituted analogue **16c**.

Nitro group reduction of **16a** using iron powder and 1N HCl afforded the arylamine **17** in 84% yield. Finally, the chloro-substituted hydrazide **9a** was formed in 27% yield from **17** *via* a Sandmeyer reaction.



Scheme 4. Reagents and conditions: (a) HNO_3 , CH_3COOH ; (b) HBTU, DIPEA, 1-aminopiperidine, CH_2Cl_2 ; (c) Fe, 1N HCl, EtOH/ H_2O ; (d) NaNO_2 , CuCl, 37% HCl, H_2O ; (e) chromatography on silica gel (CH_2Cl_2 /acetone 9/1).

From this reaction we also obtained low yields of the non-chlorinated corresponding derivative **16c**, which was hardly separable by column chromatography from the chloro analogue **9a**. According to Stoit *et al.* [17], the low yield of **9a** and the formation of **16c** at the end of the synthetic process were due to partial over reduction of **9a** to **16c** in the Sandmeyer reaction (step d of Scheme 4). However, we rather believe that the small amount of **16c** in the final product of this synthetic procedure might result from an incomplete nitration of the acid **14** (step a, Scheme 3), which produced a mixture of nitro regioisomers **15a** and **15b** containing a small quantity of residual starting material **14**, which is difficult to detect and separate by chromatography. Indeed, in our hands the byproduct **16c** was inseparable from **16a** under the chromatographic condition used by Stoit *et al.* [17]. Thus, **16c** was recovered unchanged together with **9a** after steps c and d of Scheme 4. The product derived from the Sandmeyer reaction was purified by chromatography on silica gel to separate **9a** from the by-product **16c**.

Comparison of the physicochemical properties and spectral data (see Experimental Section) for the new two samples of **NESS0327** obtained as illustrated above confirms their structure, which is consistent with that of the hydrazide **9a**.

3. Biology

3.1. Radioreceptor binding assays

Affinities at CB_1 and CB_2 receptors for **9a**, the corresponding closest analogues **9b-i**, and the derivatives **10a**, **10b**, **12**, and **13** were assessed by competition for [^3H]CP-55,940 binding in mouse whole brain membranes and Chinese hamster ovary (CHO) cell membranes transfected with hCB_2 , respectively [20]. The experimental data (IC_{50} values) were converted into K_i

values [21]. The results from the in vitro binding assays were compared with the K_i values of the lead cannabinoid ligands CP-55,940 and rimonabant.

3.2. Intrinsic activity by in vitro assays

According to previous papers [22-23], CB1 intrinsic activity of selected synthesized derivatives was evaluated through in vitro tests based on the determination of phosphorylated ERK 1/2 (P-ERK 1/2) expression in Mouse Neuroblastoma N1E-115 cell line. In fact, it was reported that this cell line expresses CB₁ but not CB₂ receptors, and its exposure to the cannabinoid agonists ACEA and WIN55-212,2 induces a rapid phosphorylation and activation of the ERK 1/2 [22]. This effect is counteracted by a pre-treatment of N1E-115 cells with CB₁ antagonist derivatives [22-23].

4. Results and discussion

4.1. Cannabinoid receptor affinities

The results from binding assays of **9a** obtained by both the synthetic procedures were compared with those of the two reference compounds CP-55,940 and rimonabant, as well as with the previously reported K_i data of the corresponding compounds (Table 1) [12,17-18].

Table 1

Cannabinoid Receptor Affinities of **9a**, CP-55,940, and rimonabant.

Compound	K_i CB ₁ (nM) ^a	K_i CB ₂ (nM) ^b
9a from Procedure A	4.20 (0.3-56) ^c	55.7 (18-176) ^c
9a from Procedure B	8.18 (0.9-60) ^c	85.2 (35-208) ^c
9a [12]	0.00035±0.000005 ^d	21±0.5 ^d
9a [17]	126	not reported
9a [18]	18.4±1.62 ^d	758
CP-55,940	0.62 (0.2-1.9) ^c	9.73 (1.1-18.0) ^c
rimonabant	2.82 (0.9-9.8) ^c	1400 (750-2350) ^c
rimonabant [12]	1.80±0.075 ^d	514±30 ^d

^a Affinity of compounds for the CB₁ receptor was evaluated using [³H]-CP 55,940 and mouse whole brain membranes. K_i values were obtained from five independent experiments carried out in triplicate.

^b Affinity of compounds for the CB₂ receptor was evaluated using [³H]-CP 55,940 and Chinese hamster ovary (CHO) cell membranes transfected with hCB₂ receptors. K_i values were obtained from five independent experiments carried out in triplicate.

^c 95% confidence limits.

^d literature data expressed as ±SEM.

The CB₁ affinity value of the novel sample of **9a** obtained through Procedure A (K_i CB₁ = 4.20 nM) disagrees with that previously reported by part of our group

[12-13], and it is more in accordance with the data reported by Zhang *et al.* [18]. The new nanomolar K_iCB_1 value for **9a** was confirmed also for the compound obtained from Procedure B after elimination of the by-product 1-(2',4'-dichlorophenyl)-*N*-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide **16c**, although a slightly difference was highlighted for the two K_iCB_1 values: $K_iCB_1 = 4.20$ and 8.18 nM for **9a** obtained through Procedure A and B, respectively. However, it is important to note that the new K_iCB_1 values of **9a** were at least one order of magnitude lower than that previously measured by Stoit *et al.* (K_iCB_1 126 nM) [17-18].

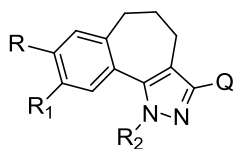
Although a different receptor matrix has been adopted for CB_2 affinity evaluation in this work, the new determined values of K_iCB_2 were comparable to that previously reported for **NESS0327** [12], with K_iCB_2 in the order of 50 nM.

CB_1 and CB_2R affinities of **9a** were compared to those determined for derivatives **9b-i**, as well as for compounds **10a-b**, and **12-13** (Table 2). As for **9a**, also the re-synthesized derivatives **9b-d**, evidenced higher K_iCB_1 values relative to previously reported data, which were in the order of 0.001-0.3 nM [13]. All the investigated **9a** closest analogues **9b-h**, evidenced K_iCB_1 data in the range of 3.6-24.8 nM, in line with the novel value determined for the lead compound. Thus, amongst the synthesized derivatives, CB_1 affinity was not substantially affected by the replacement of hydrazide groups of **9a-c** with carboxamide groups containing cycloalkyl or methylene-cycloalkyl moieties (compounds **9d-h**). The introduction of a methylene spacer between cyclohexyl ring and carboxamide group at 3 position of the pyrazole ring (from **9d** to **9e**) determined a doubling of K_iCB_1 , although this modification maintained good CB_1 affinity (**9e**: $K_iCB_1 = 19.4$ nM). No significant effect was recorded due to the replacement of cyclohexyl ring in **9e** to give derivatives **9f-h**. However, the compound bearing the bulky myrtanil substituent (**9h**) showed the best CB_1 affinity, with a $K_iCB_1 = 3.6$ nM, which was equivalent to that determined for the lead **9a**.

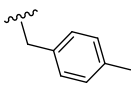
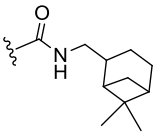
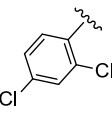
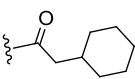
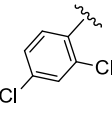
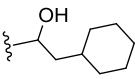
As highlighted from comparison of compounds **9h** and **9i**, the shifting of chlorine atom from 8 to 9 position of the myrtanil based tricyclic derivatives induced a decrease of CB_1 affinity. However, also derivative **9i** evidenced good CB_1 affinity (**9i**: $K_iCB_1 = 17.3$ nM). Although the comparison was limited to these two derivatives, the shifting of chlorine atom from 8 to 9 position didn't significantly affect the CB_1 affinity as instead previously showed for other analogues compounds taking into account the original K_iCB_1 value of **9a** [13].

Table 2

Structure and binding data of the synthesized compounds according to Procedure A.

**9a-i, 10a,b, 12, 13**

Compd	R	R ₁	R ₂	Q	Receptor affinity (nM) ^a		K_iCB_2/K_iCB_1
					K_iCB_1 ^b	K_iCB_2 ^c	
9a	Cl	H			4.2 (0.3-56)	55.7 (18-176)	13.26
9b	Cl	H			17.2 (1-244)	182 (95-348)	10.58
9c	Cl	H			4.5 (1-24)	28.1 (11-74)	6.24
9d	Cl	H			10.2 (1-97)	17.9 (10-31)	1.75
9e	Cl	H			19.4 (7-58)	31.8 (15-68)	1.64
9f	Cl	H			19.1 (7-49)	13.3 (6-28)	0.69
9g	Cl	H			24.8 (3-226)	52.1 (21-131)	2.10
9h	Cl	H			3.6 (2-7)	19.5 (13-30)	5.42
9i	H	Cl			17.3 (3-121)	13.6 (4-48)	0.79
10a	Cl	H			313 (28-3536)	9.5 (7-14)	0.03

10b	H	Cl			169 (48-587)	51.1 (18-95)	0.30
12	Cl	H			8.5 (6-13)	325 (147-722)	38.24
13	Cl	H			154 (97-245)	24 (7-88)	0.16

^a 95% confidence limits.

^b Affinity of compounds for the CB₁ receptor was evaluated using [³H]-CP 55,940 and mouse whole brain membranes. *K_i* values were obtained from five independent experiments carried out in triplicate.

^c Affinity of compounds for the CB₂ receptor was evaluated using [³H]-CP 55,940 and Chinese hamster ovary (CHO) cell membranes transfected with hCB₂ receptors. *K_i* values were obtained from five independent experiments carried out in triplicate.

The replacement of 2,4-dichloro-phenyl with 4-methyl-benzyl substituent at 1 position of the pyrazole ring of **9h** and **9i** determined compounds **10a** and **10b**, respectively. In both cases, the adopted modification elicited a significant reduction of CB₁ affinity, with 2-3 orders of magnitude increasing of *K_i*CB₁ values.

No significant effect on CB₁ affinity was instead observed by the introduction of a ketone group in 3 position of the pyrazole ring. Compound **12** showed in fact *K_i*CB₁ = 8.5 nM, which is equivalent to that of the closest analogues **9d**. It is important to note that a reduction of the ketone group of **12** to get derivative **13** determined a 18-fold increasing of *K_i*CB₁ value by evidencing a more favourable availability of the electrons of ketone oxygen to form hydrogen bonds with the CB₁ rather than those of the hydroxyl moiety.

Amongst the synthesized compounds, **12** evidenced the best CB₁ selectivity, with *K_i*CB₂/*K_i*CB₁ = 38.24 (Table 2). In contrast to previously reported data [12-13] and according to the novel determined *K_i*CB₁ values and confirmation of *K_i*CB₂ values, all the other compounds didn't appear as marked selective CB₁ derivatives. Acceptable CB₁ selectivity was recorded only for **9a** and **9b** (*K_i*CB₂/*K_i*CB₁ of 13.26 and 10.58, respectively), while equivalent or lower *K_i*CB₂ relative to *K_i*CB₁ values were highlighted for the other compounds. The new determined CB₁ selectivity value of **9a** is in accordance with that reported by Zhang *et al.* [18].

4.2 Intrinsic activity by in vitro assays

Preliminary in vitro assays were carried out to evaluate CB₁ mediated intrinsic activity of the synthesized compounds, with the exception of derivatives **10a-b** and **13** having *K_i*CB₁ values higher than 150 nM.

According to rimonabant [23], compounds **9a-g** and **12** didn't affect P-ERK 1/2 expression in N1E-115 cells in the concentration range 1 nM - 10 μM

(Table 3 for data at 1 μ M; dose response data not shown). Moreover, all these derivatives inhibited the P-ERK 1/2 expression up-regulation induced by the reference cannabinoid agonist WIN55,212-2 (Table 3). These data thus confirm the previously reported CB1 antagonism profile of **9a** [12], and evidence an analogue behaviour for derivatives **9b-g** and **12**.

In contrast to the other compounds, derivatives **9h-i** bearing a myrtanil substituent at the carbamoyl moiety in 3 position of pyrazole ring enhanced P-ERK 1/2 expression in N1E-115 cells. This effect was statistically recorded starting from 1 nM concentration of the compounds. CB1 agonism profile of both the derivatives was further ascertained through the rimonabant counteraction of the elicited P-ERK 1/2 expression up-regulation by **9h-i**. Representative results regarding **9h** have been reported in Fig. 4. Equivalent behaviour has been detected for **9i** (data not shown).

Table 3

Effect of compounds **9a-g** and **12** at the dose of 1 μ M on P-ERK 1/2 expression in N1E-115 cells after 10 min of exposure (second column) and as 5 min of pretreatment before exposure of the same cell lines to the cannabinoid agonist WIN55,212-2 (25 nM) (third column). Data are expressed as a mean percentage of vehicle \pm SEM and are the results of five separate experiments. *P<0.05 vs vehicle; #P<0.05 vs WIN55,212-2 (25 nM) without CB1 antagonist pretreatment.

Compd	P-ERK 1/2 (% of vehicle)	
	Compd (1 μ M)	Compd (1 μ M) + WIN55,212-2 (25 nM)
Vehicle	100 \pm 10	185 \pm 12*
9a	111 \pm 12	115 \pm 13 [#]
9b	95 \pm 9	103 \pm 9 [#]
9c	107 \pm 10	102 \pm 12 [#]
9d	113 \pm 9	117 \pm 10 [#]
9e	100 \pm 14	109 \pm 12 [#]
9f	98 \pm 10	108 \pm 12 [#]
9g	113 \pm 11	118 \pm 8 [#]
12	115 \pm 7	105 \pm 15 [#]
rimonabant	110 \pm 8	108 \pm 7 [#]

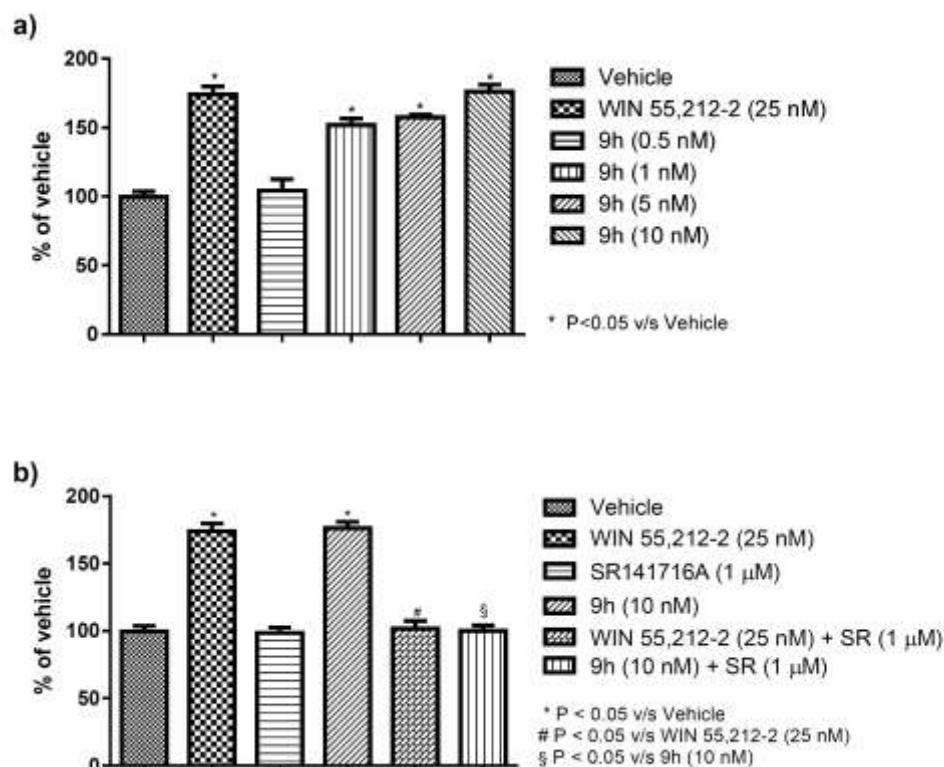


Fig. 4. a) Dose response studies of P-ERK 1/2 expression following a 10 min exposure of N1E-115 cells to different concentrations of derivative **9h** or the reference cannabinoid agonist WIN 55,212-2 (25 nM). **b)** Results of competition study of P-ERK 1/2 expression mediated by a 5 min pre-treatment of N1E-115 cells with reference CB1 antagonist/inverse agonist rimonabant (SR) followed by a 10 min exposure to WIN 55,212-2 (25 nM) or **9h** (10 nM). In **a)** and **b)** data are expressed as a mean percentage of vehicle \pm SEM (five independent experiments). A one-way Anova followed by Dunnet's multiple comparison post test was performed for statistical analysis.

5. Conclusion

We have re-synthesized ex-novo the 8-chloro-1-(2',4'-dichlorophenyl)-*N*-piperidin-1-yl-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide **9a** (NESS0327), following two alternative routes according to previously reported synthetic procedures. Comparison of the physicochemical and spectral data of the samples unequivocally demonstrates their structural equivalence. However, the procedure of Schemes 1-2 afforded the final product **9a** in higher yield and purity relative to that of Scheme 4.

The CB1 affinity values of the new samples of **9a** disagree with those previously reported by part of our group [12-13]. In accordance with the data reported by Zhang *et al.* [18], **9a** evidenced K_i CB₁ values in the order of nM units, with K_i CB₂/ K_i CB₁ = 13.26. The reasons for the discrepancy relative to our previous data are unclear. However, it doesn't appear to be due to structural differences in the synthesized compounds but rather may be related to anomalies in the procedures used in the original pharmacological testing (binding assays) of the compound. The new reported data indicate that the CB1 affinity of **9a** is

consistent with that of the lead compound rimonabant. Moreover, it is supported by the determined cannabinoid receptor profile of **9a** analogue derivatives **9b-i**.

The importance of compound **9a** and analogues remains high considering both the new CB1 affinity value and the confirmation of antagonism profile of the compound re-synthesized for this work. According to intrinsic activity assays, the substituent at carbamoyl group at 3 position of the pyrazole ring determines significant differences in CB1 modulation. In contrast to **9a** and other investigated analogues, the myrtanil carboxamides **9h,i** show in fact a biological profile consistent with that of CB₁ agonist compounds.

6. Experimental protocols

6.1. Chemistry

6.1.1. General Methods

Melting points were obtained on a Stuart SMP10 Melting point apparatus and are uncorrected. IR spectra were recorded on a Bruker Alpha-T FT-IR Spectrophotometer equipped with an Alpha Eco ATR as sampling module and are expressed in ν (cm⁻¹). All NMR spectra were taken on a Bruker Ultra Shield 400 Avance III NMR Spectrometer, with ¹H and ¹³C observed at 400 and 100 MHz, respectively. Chemical shifts for ¹H and ¹³C NMR spectra are expressed in δ downfield from TMS (Me₄Si). Multiplicities are reported as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), qu (quintuplet), dd (doublet of doublets), m (multiplet). ESI mass spectra were recorded on a Bruker Esquire 3000 Plus Ion Trap mass spectrometer in the positive mode. Combustion analyses of all target compounds were performed by Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari, Italy, and are within $\pm 0.4\%$ of the theoretical values, corresponding to $\geq 95\%$ purity. All reactions involving air or moisture sensitive compounds were performed under highly pure nitrogen atmosphere. Flash chromatography (FC) was performed using Merck silica gel 60 (230-400 mesh ASTM). Thin layer chromatography (TLC) was performed with Polygram[®] SIL N-HR/HV₂₅₄ precoated plastic sheets (0.2 mm).

Unless otherwise specified, all materials, solvents, and reagents were obtained from Sigma-Aldrich (Milan, Italy).

The requisite carboxylic acid compounds to get final derivatives were prepared as previously reported in literature. Physicochemical properties and spectral data of the synthesized intermediates were in accordance with those previously recorded [13,17,19]

6.1.2. General procedure A: preparation of carbohydrazides **9a-c** and carboxamides **9d-i**.

A mixture of the appropriate carboxylic acid **7a-b** or **8a-b** (1.0 eq, 0.25 mmol), 1-hydroxybenzotriazole hydrate (HOBt) (1.2 eq, 0.30 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (1.2 eq, 0.30 mmol) in dichloromethane (2.0 mL) was stirred at room temperature for 1 h. A solution of the requisite hydrazine or amine (2.0 eq, 0.50 mmol) in dichloromethane (3.0 mL) was added drop-wise. The resulting mixture was stirred at room temperature for 22 h. The solvent was removed under vacuum and the residue was purified by flash-chromatography to afford the desired compound.

When the starting hydrazines or amines were used as hydrochloride salt, triethylamine (TEA) (2.0 eq, 0.50 mmol) was added to the mixture.

6.1.2.1. N-Piperidin-1-yl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (9a) [12].

General procedure A was used to convert **7a** and *N*-aminopiperidine into the title product. White solid; yield: 79%; mp 199-200°C (acetone), (202 °C [12]); *R_f* 0.40 (diethyl ether/petroleum ether, 6:4); IR 3200, 1650; ESI-MS (*m/z*): 513.1 (M+Na); ¹H-NMR (CDCl₃) δ 1.40-1.48 (m, 2H), 1.72-1.80 (m, 4H), 2.19-2.30 (m, 2H), 2.66 (t, 2H, *J* = 6.6 Hz), 2.83-2.90 (m, 4H), 2.90-3.48 (m, 2H), 6.56 (d, 1H, *J* = 8.3 Hz), 7.00 (dd, 1H, *J* = 2.2 and 8.3 Hz), 7.30 (d, 1H, *J* = 2.2 Hz), 7.40 (dd, 1H, *J* = 2.2 and 8.3 Hz), 7.42-7.48 (m, 2H), 7.66 (bs, 1H); ¹³C-NMR (CDCl₃) δ 20.2, 23.4, 25.5, 31.4, 32.6, 57.1, 122.7, 126.2, 127.7, 128.0, 128.2, 129.9, 130.4, 130.5, 132.5, 134.2, 135.9, 136.0, 142.2, 143.6, 143.8, 160.0. Anal. C₂₄H₂₃Cl₃N₄O (C, H, Cl, N).

6.1.2.2. N-Pyrrolidin-1-yl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (9b) [13].

General procedure A was used to convert **7a** and *N*-aminopyrrolidine hydrochloride into the title product. Because of an excess of the hydrochloride salt, 2 eq of TEA were used in this reaction. White solid; yield: 60%; mp 127-128°C; *R_f* 0.33 (petroleum ether/EtOAc 4:6); IR 1651, 3206; ¹H-NMR (CDCl₃) δ 1.82-1.96 (m, 4H), 2.25 (t, 2H, *J* = 6.4 Hz), 2.66 (t, 2H, *J* = 6.4 Hz), 2.90-3.10 (m, 6H), 6.57 (d, 1H, *J* = 8.2 Hz), 6.99 (dd, 1H, *J_o* = 8.2 Hz, *J_m* = 2.2 Hz), 7.28-7.31 (m, 1H), 7.37-7.49 (m, 3H), 7.66 (br s, 1H, NH exch. with D₂O); ¹³C-NMR (CDCl₃) δ 20.04 (CH₂), 22.20 (CH₂ x 2), 31.32 (CH₂), 32.41 (CH₂), 55.31 (CH₂ x 2), 122.47 (C), 126.10 (CH), 127.50 (C), 127.93 (CH), 128.11 (CH), 129.81 (CH), 130.29 (CH), 130.37 (CH), 132.36 (C), 134.06 (C), 135.84 (C), 142.13 (C), 143.34 (C), 143.67 (C), 160.72 (C), 169.98 (CO); API-ES calcd for 475.8, trovato 475.10; Anal. calcd for C₂₃H₂₁Cl₃N₄O: C, 58.06; H, 4.45; Cl, 22.35; N, 11.78. Trovato: C, 58.25; H, 4.46; Cl, 22.37; N, 11.81.

6.1.2.3. N-Homopiperidin-1-yl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (9c) [13].

General procedure A was used to convert **7a** and *N*-aminohomopiperidine into the title product. White solid; yield: 35%; mp 160-161°C; *R_f* 0.54 (petroleum ether/EtOAc 6:4); IR 1659, 3174; ¹H-NMR (CDCl₃) δ 1.60-1.80 (m, 10H), 2.25 (qu, 2H, *J* = 6.0 Hz), 2.65 (t, 2H, *J* = 6.2 Hz), 3.15 (t, 4H, *J* = 5.6 Hz), 6.56 (d, 1H, *J* = 8.4 Hz), 6.99 (dd, 1H, *J_o* = 8.6 Hz, *J_m* = 1.8 Hz), 7.30 (s, 1H), 7.37-7.48 (m, 3H), 8.10 (br s, 1H, NH exch. with D₂O); ¹³C-NMR (CDCl₃) δ 20.10 (CH₂), 26.36 (CH₂), 26.95 (CH₂ x 2), 29.67 (CH₂), 31.41 (CH₂), 32.49 (CH₂), 58.32 (CH₂ x 2), 122.57 (C), 126.17 (CH), 127.62 (C), 127.10 (CH), 128.14 (CH), 129.89 (CH), 130.32 (CH), 130.46 (CH), 132.42 (C), 134.11 (C x 2), 135.86 (C), 142.17 (C), 143.74 (C x 2), 160.72 (CO); API-ES calcd for 503.9, trovato 503.01; Anal. calcd for C₂₅H₂₅Cl₃N₄O: C, 59.59; H, 5.00; Cl, 21.10; N, 11.12. Trovato: C, 59.76; H, 5.02; Cl, 21.15; N, 11.13.

6.1.2.4.N-Cyclohexyl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-

tetrahydrobenzo[6,7]cyclohepta [1,2-c]pyrazole-3-carboxamide (9d) [13].

General procedure A was used to convert **7a** and cyclohexylamine into the title product. White solid; yield: 50%; mp 96-98°C; R_f 0.54 (petroleum ether/EtOAc 8:2); IR 1633, 3201; $^1\text{H-NMR}$ (CDCl_3) δ 1.16-1.45 (m, 7H), 1.62-1.82 (m, 2H), 1.96-2.06 (m, 2H), 2.25 (qu, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 6.6$ Hz), 2.95-3.12 (m, 2H), 6.57 (d, 1H, $J = 8.4$ Hz), 6.86 (d, 1H, $J = 8.6$ Hz), 6.99 (dd, 1H, $J_o = 8.0$ Hz, $J_m = 2.0$ Hz), 7.30 (d, 1H, $J = 2.2$ Hz), 7.38-7.46 (m, 2H), 7.50 (br s, 1H, NH exch. with D_2O); $^{13}\text{C-NMR}$ (CDCl_3) δ 20.18 (CH_2), 25.01 ($\text{CH}_2 \times 2$), 25.59 (CH_2), 31.49 (CH_2), 32.50 (CH_2), 33.19 ($\text{CH}_2 \times 2$), 47.92 (CH), 122.22 (C), 126.16 (CH), 127.74 (C), 127.99 (CH), 128.17 (CH), 129.88 (CH), 130.38 (CH), 130.45 (CH), 132.46 (C), 134.05 (C), 135.85 (C), 135.98 (C), 142.28 (C), 143.73 (C), 144.25 (C), 161.77 (CO); API-ES calcd for 488.84, trovato 488.10; Anal. calcd for $\text{C}_{25}\text{H}_{24}\text{Cl}_3\text{N}_3\text{O}$: C, 61.43; H, 4.95; Cl, 21.76; N, 8.60. Trovato: C, 61.25; H, 4.94; Cl, 21.69; N, 8.58.

6.1.2.5.N-Cyclohexylmethyl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7] cyclohepta [1,2-c]pyrazole-3-carboxamide (9e).

General procedure A was used to convert **7a** and cyclohexanemethylamine into the title product. White solid; yield: 44%; R_f 0.45 (petroleum ether/ethyl acetate 9:1); IR 3410, 1670; $^1\text{H-NMR}$ (CDCl_3) δ 0.92-1.03 (m, 2H); 1.10-1.32 (m, 4H); 1.53-1.84 (m, 5H); 2.20-2.31 (m, 2H); 2.60-2.72 (m, 3H); 2.80-3.15 (m, 1H); 3.20-3.30 (m, 2H); 6.58 (d, 1H, $J = 8.0$ Hz); 6.97-7.08 (m, 2H); 7.30 (d, 1H, $J = 1.6$ Hz); 7.40 (dd, 1H, $J = 1.7$ and 8.4 Hz); 7.42-7.48 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ 20.2, 25.9, 26.4, 31.0, 31.5, 32.5, 38.1, 45.3, 122.2, 126.2, 127.8, 128.1, 128.2, 129.9, 130.4, 130.5, 132.5, 134.1, 135.9, 136.1, 142.3, 143.8, 144.2, 162.7. Anal. calc. for $\text{C}_{26}\text{H}_{26}\text{Cl}_3\text{N}_3\text{O}$: C, 62.10; H, 5.21; Cl, 21.15; N, 8.36. Trovato: C, 62.03; H, 5.20; Cl, 21.13; N, 8.34.

6.1.2.6.N-(1-Adamantylmethyl)-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7] cyclohepta[1,2-c]pyrazole-3-carboxamide (9f).

General procedure A was used to convert **7a** and 1-adamantylmethylamine into the title product. White solid; yield: 59%; R_f 0.41 (petroleum ether/ethyl acetate 9:1); IR 3405, 1669; $^1\text{H-NMR}$ (CDCl_3) δ 1.57 (bs, 6H); 1.61-1.75 (m, 6H); 1.95-2.02 (m, 3H); 2.20-2.30 (m, 2H); 2.63-2.73 (m, 3H); 2.82-3.18 (m, 3H); 6.58 (d, 1H, $J = 8.4$ Hz); 6.97-7.09 (m, 2H); 7.30 (bs, 1H); 7.40 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.44-7.50 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ 20.3, 28.3, 31.4, 32.5, 34.1, 37.0, 40.3, 50.5, 122.2, 126.2, 127.8, 128.1, 128.2, 129.9, 130.4, 130.5, 132.5, 134.1, 135.9, 136.1, 142.3, 143.8, 144.3, 162.9. Anal. calc. for $\text{C}_{30}\text{H}_{30}\text{Cl}_3\text{N}_3\text{O}$: C, 64.93; H, 5.45; Cl, 19.17; N, 7.57. Trovato: C, 64.81; H, 5.44; Cl, 19.10; N, 7.55.

6.1.2.7.N-(2,3,4,5-Tetrahydrofurfuryl)-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7] cyclohepta[1,2-c]pyrazole-3-carboxamide (9g).

General procedure A was used to convert **7a** and R-(−)-tetrahydrofurfurylamine into the title product. White solid; yield: 54%; R_f 0.38 (petroleum ether/ethyl

acetate 7:3); IR 3409, 1667; $^1\text{H-NMR}$ (CDCl_3) δ 1.58-1.74 (m, 1H); 1.84-1.96 (m, 2H); 1.97-2.08 (m, 1H); 2.19-2.31 (m, 2H); 2.61-2.72 (m, 2H); 2.75-3.28 (m, 2H); 3.31-3.41 (m, 1H); 3.66-3.81 (m, 2H); 3.84-3.94 (m, 1H); 4.04-4.12 (m, 1H); 6.57 (d, 1H, $J = 8.0$ Hz); 7.00 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.27-7.32 (m, 2H); 7.39 (dd, 1H, $J = 1.9$ and 8.4 Hz); 7.41-7.48 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ 19.7, 25.3, 28.2, 30.9, 31.9, 42.2, 67.6, 77.3, 121.6, 125.6, 126.4, 127.2, 127.5, 127.6, 129.3, 129.8, 129.9, 131.8, 133.5, 135.2, 141.6, 143.2, 143.4, 162.3. Anal. calc. for $\text{C}_{24}\text{H}_{22}\text{Cl}_3\text{N}_3\text{O}_2$: C, 58.73; H, 4.52; Cl, 21.67; N, 8.56. Trovato: C, 58.69; H, 4.51; Cl, 21.65; N, 8.54.

6.1.2.8. *N*-(Myrtanyl)-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta [1,2-*c*]pyrazole-3-carboxamide (9h).

General procedure A was used to convert **7a** and (–)-*cis*-myrtanylamine into the title product. White solid; yield: 81%; R_f 0.48 (petroleum ether/ethyl acetate 6:4); IR 3400, 1662; $^1\text{H-NMR}$ (CDCl_3) δ 1.07 (s, 3H); 1.21 (s, 3H); 1.52-1.63 (m, 5H); 1.82-2.05 (m, 4H); 2.15-2.29 (m, 4H); 2.64 (t, 2H, $J = 6.6$ Hz); 3.35-3.45 (m, 2H); 6.59 (d, 1H, $J = 8.0$ Hz); 7.00 (dd, 1H, $J = 2.2$ e 8.0 Hz); 7.25-7.32 (m, 1H); 7.37-7.43 (m, 3H); 7.51 (bt, 1H, $J = 5.4$ Hz); $^{13}\text{C-NMR}$ (CDCl_3) δ 19.86 (CH_3), 20.20 (CH_3), 23.23 (CH_2), 26.02 (CH_2), 27.96 (CH_2), 31.44 (CH_2), 33.49 (CH_2), 33.28 (CH_2), 38.70 (C), 41.32 (CH), 41.48 (CH), 43.81 (CH), 44.58 (CH_2), 122.15 (C), 126.16 (CH), 127.68 (CH), 128.01 (CH), 128.15 (CH), 129.88 (CH), 130.33 (CH), 130.45 (CH), 132.45 (C), 134.05 (C), 135.85 (C), 135.97 (C), 142.27 (C), 143.72 (C), 144.15 (C), 162.61 (C=O). Anal. calc. for $\text{C}_{29}\text{H}_{30}\text{Cl}_3\text{N}_3\text{O}$: C, 64.15; H, 5.57; Cl, 19.59; N, 7.74. Trovato: C, 64.09; H, 5.55; Cl, 19.56; N, 7.72.

6.1.2.9. *N*-(Myrtanyl)-9-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta [1,2-*c*]pyrazole-3-carboxamide (9i).

General procedure A was used to convert **7b** and (–)-*cis*-myrtanylamine into the title product. White solid; yield: 87%; R_f 0.50 (petroleum ether/ethyl acetate 6:4); IR 3405, 1664; $^1\text{H-NMR}$ (CDCl_3) δ 1.08 (s, 3H); 1.20 (s, 3H); 1.53-1.61 (m, 5H); 1.84-2.03 (m, 4H); 2.19-2.30 (m, 4H); 2.63 (t, 2H, $J = 6.4$ Hz); 3.37-3.46 (m, 2H); 6.61 (s, 1H); 6.93 (bt, 1H, $J = 5.5$ Hz); 6.98-7.10 (m, 1H); 7.17-7.23 (m, 2H); 7.49-7.55 (m, 2H); $^{13}\text{C-NMR}$ (CDCl_3) δ 19.3, 19.6, 22.6, 25.5, 27.4, 31.0, 31.5, 32.7, 38.1, 40.8, 41.0, 43.3, 44.0, 122.0, 126.2, 127.6, 127.8, 129.8, 129.9, 130.2, 130.5, 131.1, 132.0, 135.3, 135.4, 139.7, 141.5, 143.6, 162.0. Anal. calc. for $\text{C}_{29}\text{H}_{30}\text{Cl}_3\text{N}_3\text{O}$: C, 64.15; H, 5.57; Cl, 19.59; N, 7.74. Trovato: C, 64.03; H, 5.56; Cl, 19.57; N, 7.73.

6.1.2.10. *N*-(Myrtanyl)-8-chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxamide (10a).

General procedure A was used to convert **8a** and (–)-*cis*-myrtanylamine into the title product. White solid; yield: 56%; R_f 0.47 (petroleum ether/ethyl acetate 7:3); IR 3405, 1658; $^1\text{H-NMR}$ (CDCl_3) δ 1.08 (s, 3H); 1.21 (s, 3H); 1.50-1.70 (m, 3H); 1.90-2.02 (m, 4H); 2.03-2.50 (m, 9H); 2.83-2.95 (m, 2H); 3.38-3.50 (m, 2H); 5.37 (s, 2H); 6.85-7.31 (m, 8H); $^{13}\text{C-NMR}$ (CDCl_3) δ 19.90 (CH_3), 21.07 (CH_3), 21.35 (CH_3), 23.24 (CH_2), 26.04 (CH_2), 28.01 (CH_2), 30.75 (CH_2), 33.48 (CH_2), 33.27

(CH₂), 38.73 (C), 41.37 (CH), 41.61 (CH), 43.93 (CH), 44.97 (CH₂), 52.09 (CH₂), 115.75 (C), 116.40 (C), 125.65 (CH), 126.72 (CH x 2), 127.13 (CH x 2), 127.38 (CH), 129.55 (CH), 132.25 (C), 133.33 (C), 133.64 (C), 137.61 (C), 138.75 (C), 146.57 (C), 164.70 (C=O). Anal. calc. for C₃₁H₃₆ClN₃O: C, 74.16; H, 7.23; Cl, 7.06; N, 8.37. Trovato: C, 74.00; H, 7.22; Cl, 7.04; N, 8.35.

6.1.2.11. N-(Myrtanyl)-9-chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (10b).

General procedure A was used to convert **8b** and (–)-*cis*-myrtanylamine into the title product. White solid; yield: 60%; R_f 0.48 (petroleum ether/ethyl acetate 7:3); IR 3408, 1659; ¹H-NMR (CDCl₃) δ 1.15 (s, 3H); 1.19 (s, 3H); 1.90-2.14 (m, 11H); 2.31 (s, 3H); 2.42-2.46 (m, 2H); 2.85-2.89 (m, 2H); 3.39-3.45 (m, 2H); 5.38 (s, 2H); 6.92-7.22 (m, 8H). Anal. calc. for C₃₁H₃₆ClN₃O: C, 74.16; H, 7.23; Cl, 7.06; N, 8.37. Trovato: C, 74.11; H, 7.21; Cl, 7.04; N, 8.36.

6.1.3. General procedure for the synthesis of the carboxylic acids 7a, 7b, 8a, and 8b (Procedure A1).

To a mixture of appropriate ester **5a-b** and **6a-b** (1.12 mmol) in methanol (5 mL) was added a solution of potassium hydroxide (0.13 g, 2.25 mmol) in methanol (4 mL). The resulting mixture was heated under reflux overnight. The mixture was allowed to cool to room temperature and then poured into water and acidified with 1 N HCl. The precipitate was filtered, washed with water, and dried to yield the title products.

6.1.3.1. Synthesis of 8-Chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid (7a) [13].

General Procedure A1 was adopted to convert the ethyl ester **5a** into the corresponding carboxylic acid **7a**. White solid; yield 96%; R_f 0.41 (CHCl₃/MeOH 9:1); mp 270°C; IR 1690, 3410; ¹H NMR (CDCl₃) δ 2.25-2.30 (m, 2H), 2.68 (t, 2H, *J* = 6.4 Hz), 3.10-3.23 (m, 2H), 4.50 (bs, 1H), 6.61 (d, 1H, *J* = 8.4 Hz), 7.03 (dd, 1H, *J* = 2.2 and 8.2 Hz), 7.32 (d, 1H, *J* = 2.0 Hz), 7.39-7.44 (m, 2H), 7.52 (d, 1H, *J* = 8.0 Hz). Anal. (C₁₉H₁₃Cl₃N₂O₂) C, H, Cl, N.

6.1.3.2. Synthesis of 9-Chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid (7b) [13].

General Procedure A1 was adopted to convert the ethyl ester **5b** into the corresponding carboxylic acid **7b**. White solid; yield 98%; R_f 0.60 (CHCl₃/MeOH 9:1); mp 250°C; IR 1716, 3419; ¹H NMR (CDCl₃) δ 2.25-2.28 (m, 2H), 2.68 (t, 2H, *J* = 6.4 Hz), 3.08-3.30 (m, 2H), 4.80 (br s, 1H, OH exchange with D₂O), 6.63 (d, 1H, *J* = 1.8 Hz), 7.20-7.30 (m, 2H), 7.40-7.52 (m, 2H), 7.59 (d, 1H, *J* = 9.0 Hz). Anal. (C₁₉H₁₃Cl₃N₂O₂) C, H, Cl, N.

6.1.3.3. Synthesis of 8-Chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid (8a) [19].

General Procedure A1 was adopted to convert the ethyl ester **6a** into the corresponding carboxylic acid **8a**. White solid; yield 92%; IR 1716, 3413; ¹H

NMR (CDCl₃) δ 2.15-2.35 (m, 2H), 2.38 (s, 3H), 2.67 (t, 2H, J = 6.8 Hz), 3.05-3.30 (m, 2H), 3.55 (s, 2H), 6.57 (d, 1H, J = 8.1 Hz), 7.00 (dd, 1H, J = 2.0 and 8.1 Hz), 7.20 (d, 1H, J = 2.0 Hz), 7.22-7.31 (m, 4H). Anal. (C₂₁H₁₉ClN₂O₂) C, H, Cl, N.

6.1.3.4. Synthesis of 9-Chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid (8b) [19].

General Procedure A1 was adopted to convert the ethyl ester **6b** into the corresponding carboxylic acid **8b**. White solid; yield 96%; R_f 0.36 (petroleum ether /AcOEt 8.5:1.5); IR 1716, 3420; ¹H NMR (CDCl₃) δ 2.16-2.36 (m, 2H), 2.38 (s, 3H), 2.65 (t, 2H, J = 6.8 Hz), 3.05-3.34 (m, 2H), 3.56 (s, 2H), 6.61 (d, 1H, J = 8.1 Hz), 7.00 (dd, 1H, J = 2.2 and 8.1 Hz), 7.21 (d, 1H, J = 2.1 Hz), 7.24-7.34 (m, 4H). Anal. (C₂₁H₁₉ClN₂O₂) C, H, Cl, N.

6.1.4. General procedure for the synthesis of the ethyl carboxylates 5a, 5b, 6a, and 6b (General Procedure A2).

A stirred mixture of diketoeester **2a** or **2b** (0.41 g, 1.39 mmol) and 2,4-dichlorophenylhydrazine hydrochloride **3** or 4-methylbenzylhydrazine hydrochloride **4** (1.81 mmol) in EtOH (9 mL) was heated under reflux for 14 h. The mixture was allowed to cool to room temperature, and the solvent was removed under reduced pressure to give a solid, which was purified by flash chromatography (petroleum ether/EtOAc, 8.5:1.5) to afford the title products.

6.1.4.1. Synthesis of Ethyl 8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylate (5a) [13].

General Procedure A2 was used to convert **2a** and **3** into the title product. Yellow solid; yield 80%; R_f 0.48 (petroleum ether/EtOAc, 8.5:1.5); mp 158-160°C (trituated with petroleum ether), IR 1724; ¹H NMR (CDCl₃) δ 1.43 (t, 3H, J = 7.2 Hz), 2.20-2.36 (m, 2H), 2.66 (t, 2H, J = 6.4 Hz), 3.10-3.30 (m, 2H), 4.45 (q, 2H, J = 7.2 Hz), 6.60 (d, 1H, J = 8.4 Hz), 7.02 (dd, 1H, J = 2.2 and 8.4 Hz), 7.31 (d, 1H, J = 1.8 Hz), 7.37-7.42 (m, 2H), 7.54 (d, 1H, J = 9.2 Hz). Anal. (C₂₁H₁₇Cl₃N₂O₂) C, H, Cl, N.

6.1.4.2. Synthesis of Ethyl 9-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylate (5b) [13].

General Procedure A2 was used to convert **2b** and **3** into the title product. Yellow solid; yield 70%; R_f 0.43 (petroleum ether/EtOAc, 9:1); mp 176-178°C (trituated with petroleum ether), IR 1710; ¹H NMR (CDCl₃) δ 1.43 (t, 3H, J = 7.0 Hz), 2.12-2.36 (m, 2H), 2.66 (t, 2H, J = 6.8 Hz), 3.10-3.36 (m, 2H), 4.44 (q, 2H, J = 7.2 Hz), 6.65 (s, 1H), 7.12-7.28 (m, 2H), 7.35-7.48 (m, 2H), 7.57 (d, 1H, J = 9.0 Hz). Anal. (C₂₁H₁₇Cl₃N₂O₂) C, H, Cl, N.

6.1.4.3. Synthesis of Ethyl 8-chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazol-3-carboxylate (6a) [19].

General Procedure A2 was used to convert **2a** and **4** into the title product. Yellow solid; yield 75%; R_f 0.42 (petroleum ether/EtOAc, 8.5:1.5); IR 1725; ¹H NMR

(CDCl₃) δ 1.40 (t, 3H, J = 7.1 Hz), 2.18-2.36 (m, 2H), 2.36 (s, 3H), 2.66 (t, 2H, J = 6.6 Hz), 3.07-3.34 (m, 2H), 3.55 (s, 2H), 4.44 (q, 2H, J = 7.1 Hz), 6.61 (d, 1H, J = 8.2 Hz), 6.98 (dd, 1H, J = 2.2 and 8.1 Hz), 7.22 (d, 1H, J = 2.1 Hz), 7.22-7.30 (m, 4H). Anal. (C₂₃H₂₃ClN₂O₂) C, H, Cl, N.

6.1.4.4. Synthesis of Ethyl 9-chloro-1-(4'-methylbenzyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazol-3-carboxylate (6b**) [19].**

General Procedure A2 was used to convert **2b** and **4** into the title product. Yellow solid; yield 73%; R_f 0.40 (petroleum ether/EtOAc, 8.5:1.5); IR 1720; ¹H NMR (CDCl₃) δ 1.42 (t, 3H, J = 7.2 Hz), 2.16-2.38 (m, 2H), 2.36 (s, 3H), 2.64 (t, 2H, J = 6.7 Hz), 3.05-3.32 (m, 2H), 3.56 (s, 2H), 4.45 (q, 2H, J = 7.2 Hz), 6.61 (d, 1H, J = 8.0 Hz), 7.00 (dd, 1H, J = 2.0 and 8.0 Hz), 7.24 (d, 1H, J = 2.0 Hz), 7.24-7.32 (m, 4H). Anal. (C₂₃H₂₃ClN₂O₂) C, H, Cl, N.

6.1.5. Preparation of N-methoxy-N-methyl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (11**).**

Trimethylaluminum (0.92 ml of 2 M solution in hexane, 1.84 mmol) was added dropwise to a suspension of dimethylhydroxylamine hydrochloride (0.18 g, 1.84 mmol) in CH₂Cl₂ (3 ml) at 0 °C. The solution was stirred at 0 °C for 45 min and then at room temperature for 40 min. To this solution, the compound **5a** (0.4 g, 0.92 mmol) in CH₂Cl₂ (2 ml) was added dropwise. Stirring was continued for 4 h at room temperature. The reaction mixture was cooled to 0 °C, and 10% HCl was carefully added dropwise. The mixture was extracted with CH₂Cl₂, washed with water, brine, dried over Na₂SO₄, and filtered. The residue after evaporation of the solvent was purified by flash chromatography (petroleum ether/ethyl acetate 7:3) obtaining 0.33 g (80% yield) of compound **11** as a white solid. R_f 0.38 (petroleum ether/ethyl acetate 7:3); IR 1681; ¹H-NMR (CDCl₃) δ 2.21-2.30 (m, 2H); 2.64-2.75 (m, 4H); 3.46 (s, 3H); 3.80 (s, 3H); 6.60 (d, 1H, J = 8.3 Hz); 7.02 (dd, 1H, J = 2.2 and 8.3 Hz); 7.30 (d, 1H, J = 1.6 Hz); 7.36 (dd, 1H, J = 2.2 and 8.3 Hz); 7.40 (d, 1H, J = 8.3 Hz); 7.45 (d, 1H, J = 2.2 Hz). Anal. calc. for C₂₁H₁₈Cl₃N₃O₂: C, 55.96; H, 4.03; Cl, 23.60; N, 9.32. Trovato: C, 55.82; H, 4.02; Cl, 23.57; N, 9.30.

6.1.6. Preparation of 8-chloro-1-(2',4'-dichlorophenyl)-3-(1-oxo-2-cyclohexyleth-1-yl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole (12**).**

To a solution of **11** (0.29 g, 0.64 mmol) in THF (6 ml) was added dropwise the (cyclohexylmethyl)magnesium bromide (3.86 ml of 0.5 M solution in THF, 1.93 mmol) at 0 °C under nitrogen. The reaction mixture was gradually warmed to room temperature and stirred at the same temperature for 24 h. Saturated NH₄Cl was added dropwise at 0 °C, the reaction mixture was diluted with EtOAc, and the layers were separated. The aqueous layer was extracted with EtOAc, and the combined organic layers were washed with water, dried (Na₂SO₄), and filtered. The residue after evaporation of the solvent was purified by flash chromatography (petroleum ether/diethyl ether 9:1) obtaining 80 mg (26% yield) of compound **12** as a white solid. R_f 0.56 (petroleum ether/diethyl ether 9:1); IR 1685; ¹H-NMR (CDCl₃) δ 0.98-1.11 (m, 2H); 1.13-1.38 (m, 4H); 1.62-1.81 (m, 4H); 1.97-2.10 (m, 1H); 2.18-2.29 (m, 2H); 2.62-2.71 (m, 2H); 2.85-3.18 (m, 4H); 6.58 (d, 1H, J

= 8.3 Hz); 7.00 (dd, 1H, J = 2.2 and 8.3 Hz); 7.30 (d, 1H, J = 2.2 Hz); 7.40 (dd, 1H, J = 2.2 and 8.3 Hz); 7.44-7.48 (m, 2H); ¹³C-NMR (CDCl₃) δ 25.3, 26.0, 26.2, 29.0, 32.9, 34.1, 37.4, 68.2, 121.7, 125.6, 125.7, 127.5, 127.6, 129.3, 129.4, 129.8, 129.9, 130.0, 132.0, 201.9. Anal. calc. for C₂₆H₂₅Cl₃N₂O: C, 64.01; H, 5.17; Cl, 21.80; N, 5.74. Trovato: C, 63.89; H, 5.16; Cl, 21.77; N, 5.72.

6.1.7. Preparation of 8-chloro-1-(2',4'-dichlorophenyl)-3-(1-hydroxy-2-cyclohexyleth-1-yl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole (13).

To a suspension of ketone **12** (60 mg, 0.12 mmol) in MeOH (3 ml) was added sodium borohydride (10 mg, 0.25 mmol), and the mixture was stirred at room temperature for 1.5 h. The reaction mixture was diluted with CHCl₃ and washed with water. The organic layer was dried over anhydrous sodium sulfate, filtered, and concentrated to obtain 60 mg (99% yield) of compound **13** as a white solid. R_f 0.34 (petroleum ether/ethyl acetate 8:2); IR 3315; ¹H-NMR (CDCl₃) δ 0.84-1.05 (m, 2H); 1.08-1.24 (m, 4H); 1.34-1.78 (m, 6H); 1.80-1.90 (m, 2H); 2.14-2.26 (m, 2H); 2.46-2.72 (m, 4H); 5.00 (bs, 1H); 6.60 (d, 1H, J = 8.3 Hz); 7.01 (dd, 1H, J = 2.2 and 8.3 Hz); 7.29 (d, 1H, J = 2.2 Hz); 7.35 (dd, 1H, J = 2.2 and 8.9 Hz); 7.40-7.45 (m, 2H); ¹³C-NMR (CDCl₃) δ 20.1, 26.2, 26.4, 26.6, 31.7, 32.5, 33.0, 34.1, 34.3, 45.5, 117.5, 126.2, 128.0, 128.1, 128.4, 129.8, 130.3, 130.5, 132.7, 133.7, 135.3, 136.5, 143.3, 154.3. Anal. calc. for C₂₆H₂₇Cl₃N₂O: C, 63.75; H, 5.56; Cl, 21.71; N, 5.72. Trovato: C, 63.68; H, 5.55; Cl, 21.69; N, 5.71.

6.1.8. Synthesis of N-piperidin-1-yl-8-chloro-1-(2',4'-dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (9a) [17] and N-piperidin-1-yl-1-(2',4'-dichlorophenyl)-1,4,5,6-

tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (16c), procedure B [18]. Iron powder (1.30 g, 23.28 mmol) was suspended in a mixture of water (15 ml) and ethanol (5.57 ml). 1 N HCl (0.82 ml) was added and the resulting mixture was stirred at 50 °C. After addition of **16a** (1.08 g, 2.16 mmol) the mixture was heated for 3 h at 65 °C. After cooling to room temperature ethyl acetate and water were added and the iron-containing precipitate was removed by filtration over Celite. The ethyl acetate layer was separated, washed with water, dried over Na₂SO₄, filtered and concentrated. The residue was purified by chromatography on silica gel eluting with CH₂Cl₂/acetone 4/1 to give the amine intermediate **17** (0.85 g, 84%) as a yellow solid [18]. Addition of **17** (0.79 g, 1.68 mmol) to cooled concentrated HCl (13 ml) was followed by addition at 0 °C of a solution of NaNO₂ (0.12 g, 1.74 mmol) in water (1 ml). This solution was stirred at 0 °C for 30 min and quickly added to a cooled solution of CuCl (85 mg, 0.86 mmol) in concentrated HCl (2 ml). The resulting mixture was heated at 55 °C for 1 h, cooled to room temperature, followed by addition of ice and ethyl acetate. A mixture of ice and concentrated NaOH was added. The ethyl acetate layer was separated and the water layer extracted with ethyl acetate. The combined ethyl acetate layers were filtered over Celite, washed with water, dried over Na₂SO₄, filtered and concentrated. The residue was purified by chromatography on silica gel eluting with CH₂Cl₂/acetone 9/1 to afford **9a** (0.22 g, 27%) and **16c** (50 mg, 6%) as white solids.

9a: mp 198-200°C, (202°C [12]). R_f 0.40 (diethyl ether/petroleum ether, 6:4); IR (nujol): 3210, 1650; ^1H NMR (CDCl_3) δ 1.40-1.48 (m, 2H), 1.72-1.80 (m, 4H), 2.19-2.30 (m, 2H), 2.66 (t, 2H, $J = 6.6$ Hz), 2.83-2.90 (m, 4H), 2.90-3.48 (m, 2H), 6.56 (d, 1H, $J = 8.3$ Hz), 7.00 (dd, 1H, $J = 2.2$ and 8.3 Hz), 7.30 (d, 1H, $J = 2.2$ Hz), 7.40 (dd, 1H, $J = 2.2$ and 8.3 Hz); 7.42-7.48 (m, 2H), 7.66 (bs, 1H); ^{13}C NMR (CDCl_3) δ 20.2, 23.4, 25.5, 31.4, 32.6, 57.1, 122.7, 126.2, 127.7, 128.0, 128.2, 129.9, 130.4, 130.5, 132.5, 134.2, 135.9, 136.0, 142.2, 143.6, 143.8, 160.0. Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_3\text{N}_4\text{O}$ (C, H, Cl, N).

16c: mp 165-168 °C (167-169°C [18]). R_f 0.41 (CH_2Cl_2 / diethyl ether 9/1); IR (nujol): 3165, 1650; ^1H NMR (CDCl_3) δ 1.39-1.48 (m, 2H), 1.70-1.79 (m, 4H), 2.21-2.31 (m, 2H), 2.68 (t, 2H, $J = 6.6$ Hz), 2.85-2.92 (m, 4H), 3.00-3.30 (m, 2H), 6.64 (bd, 1H, $J = 7.7$ Hz), 7.02 (td, 1H, $J = 1.3$ and 7.6 Hz), 7.21 (td, 1H, $J = 1.3$ and 7.5 Hz), 7.30 (bd, 1H, $J = 6.9$ Hz), 7.39 (dd, 1H, $J = 2.2$ and 8.4 Hz), 7.44 (d, 1H, $J = 2.2$ Hz), 7.46 (d, 1H, $J = 8.4$ Hz), 7.69 (bs, 1H). Anal. ($\text{C}_{24}\text{H}_{24}\text{Cl}_2\text{N}_4\text{O}$) C, H, Cl, N.

6.1.8.1. Synthesis of N-Piperidin-1-yl-1-(2',4'-dichlorophenyl)-8-nitro-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (16a) and N-Piperidin-1-yl-1-(2',4'-dichlorophenyl)-9-nitro-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxamide (16b) [17]

To a suspension of a mixture of **15a** and **15b** (2.80 g, 6.69 mmol) in dry acetonitrile (53 ml) is successively added *N,N*-diisopropylethylamine (DIPEA) (2.56 ml, 14.72 mmol), *O*-(Benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) (3.04 g, 8.03 mmol) and 1-aminopiperidine (0.87 ml, 8.03 mmol) and the resulting mixture is stirred at room temperature overnight. The mixture is concentrated *in vacuo* and the residue is dissolved in ethyl acetate, washed with water, dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by chromatography on silica gel eluting with diethyl ether, to afford 1.11 g of the desired carboxamide **16a** as a white solid (33% yield) and 0.44 g of **16b** (13% yield) as white solid.

16a: mp 178-180°C. R_f 0.77 (diethyl ether); ^1H NMR (CDCl_3) δ 1.41-1.49 (m, 2H), 1.71-1.81 (m, 4H), 2.25-2.38 (m, 2H), 2.81 (t, 2H, $J = 6.6$ Hz), 2.83-2.92 (m, 4H), 3.20-3.60 (m, 2H), 6.80 (d, 1H, $J = 8.5$ Hz), 7.42-7.48 (m, 2H), 7.52 (d, 1H, $J = 9.0$ Hz), 7.67 (bs, 1H), 7.89 (dd, 1H, $J = 2.3$ and 8.5 Hz), 8.18 (d, 1H, $J = 2.3$ Hz). Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_3$ (C, H, Cl, N).

16b: mp 200-202°C. R_f 0.66 (diethyl ether); ^1H NMR (CDCl_3) δ 1.40-1.49 (m, 2H), 1.72-1.81 (m, 4H), 2.22-2.41 (m, 2H), 2.80 (t, 2H, $J = 6.8$ Hz), 2.84-2.90 (m, 4H), 3.19-3.58 (m, 2H), 7.43 (d, 1H, $J = 2.2$ Hz), 7.45-7.52 (m, 2H), 7.55 (d, 1H, $J = 2.3$ Hz), 7.59 (d, 1H, $J = 8.5$ Hz), 7.68 (bs, 1H), 8.06 (dd, 1H, $J = 2.3$ and 8.5 Hz). Anal. $\text{C}_{24}\text{H}_{23}\text{Cl}_2\text{N}_5\text{O}_3$ (C, H, Cl, N).

6.1.8.2. Synthesis of the mixture of 1-(2',4'-Dichlorophenyl)-8-nitro-1,4,5,6-tetrahydrobenzo[6,7] cyclohepta[1,2-c]pyrazole-3-carboxylic acid (15a) and 1-(2',4'-dichlorophenyl)-9-nitro-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-c]pyrazole-3-carboxylic acid (15b) [17].

Fuming nitric acid (1.5 mL) is slowly added to acetic acid (1.5 mL) cooled at 0 °C.

1-(2',4'-Dichlorophenyl)-1,4,5,6-tetrahydrobenzo[6,7]cyclohepta[1,2-*c*]pyrazole-3-carboxylic acid (**14**) (0.50 g, 1.34 mmol) is added and the resulting mixture is stirred at room temperature overnight and poured onto ice. The formed precipitate is collected by filtration, washed with water and dried to give crude nitration product (98% yield) as white solid which consists of the 8-nitro **15a** and 9-nitro **15b** regioisomers in a molar ratio of 5 : 3. *R_f* 0.38 (CHCl₃/MeOH 9:1); ¹H NMR (DMSO-*d*₆) (**15a**) δ 2.03-2.18 (m, 2H), 3.09-3.30 (m, 4H), 6.95 (d, 1H, *J* = 8.6 Hz), 7.67 (dd, 1H, *J* = 2.2 and 8.5 Hz), 7.81 (d, 1H, *J* = 2.2 Hz), 7.86 (d, 1H, *J* = 8.5 Hz), 7.97 (dd, 1H, *J* = 2.3 and 8.6 Hz), 8.29 (d, 1H, *J* = 2.2 Hz), 12.96 (bs, 1H). ¹H NMR (DMSO-*d*₆) (**15b**) δ 2.04-2.19 (m, 2H), 3.09-3.31 (m, 4H), 7.52 (d, 1H, *J* = 2.3 Hz), 7.68 (d, 1H, *J* = 8.5 Hz), 7.71 (dd, 1H, *J* = 2.2 and 8.6 Hz), 7.83 (d, 1H, *J* = 2.2 Hz), 7.92 (d, 1H, *J* = 8.5 Hz), 8.10 (dd, 1H, *J* = 2.4 and 8.4 Hz), 13.05 (bs, 1H).

6.2. Biological assays.

6.2.1. Radioreceptor binding assays.

6.2.1.1. *General procedures binding experiments.* Affinities at CB₁ and CB₂ receptors for **9a** were assessed by competition for [³H]CP-55,940 binding in mouse whole brain membranes and Chinese hamster ovary (CHO) cell membranes transfected with hCB₂, respectively [20]. The results from the in vitro binding assays were compared with the *K_i* values of the prototypical cannabinoid ligands CP-55,940 and rimonabant.

6.2.1.2. *Mouse Brain Membranes [19].* Whole mouse brains from four adult male MF1 mice were suspended in centrifugation buffer (320 mM sucrose, 2 mM Tris·HCl, 2 mM Tris base, 2 mM EDTA, 5 mM MgCl₂ at pH 7.4) and the tissue homogenized with an Ultra-Turrex homogenizer. Tissue homogenates were centrifuged at 1600 x *g* for 10 min and the resulting supernatant collected. The pellet was resuspended in centrifugation buffer, centrifuged as before and the supernatant collected. Supernatants were combined before undergoing further centrifugation at 28,000 x *g* for 20 min. The supernatant was discarded and the pellet resuspended in 20 ml of buffer A (50 mM Tris·HCl, 50 mM Tris base, 2 mM EDTA, 5 mM MgCl₂ at pH 7.0) and incubated at 37 °C for 10 min. Following the incubation, the suspension was centrifuged for 20 min at 23,000 x *g*. After resuspending the pellet in another 20 ml of buffer A, the suspension was incubated for 40 min at room temperature before a final centrifugation for 15 min at 11,000 x *g*. The final pellet was resuspended in 2 ml of buffer B (50 mM Tris·HCl, 50 mM Tris base, 1 mM EDTA, 3 mM MgCl₂ at pH 7.4) to give a protein concentration of 1 mg/ml and stored at -80 °C. All centrifugation procedures were carried out at 4 °C.

6.2.1.3. *CHO cell membranes [20].* CHO cells stably transfected with cDNA encoding human cannabinoid CB₂ receptors (*B_{max}* = 72.6 pmol/mg protein) were maintained at 37 °C and 5% CO₂ in Dulbecco's Modified Eagles's medium (DMEM) nutrient mixture F-12 HAM supplemented with 10% Foetal Bovine

Serum (FBS), 3 ml penicillin–streptomycin and 4 ml G-418. These CHO-hCB₂ cells were passed twice a week using a nonenzymatic cell dissociation solution (5ml EDTA). The hCB₂ transfected cells were removed from flasks by scraping and then frozen as a pellet at –20 °C until required. Before use in a radioligand-binding assay, cells were defrosted, diluted in 50mM Tris-binding buffer (see radioligand displacement assay), and homogenized with a 1ml hand-held homogenizer. Protein assays were performed using a Bio-Rad Dc kit.

6.2.1.4. Radioligand displacement assay [20]. Binding experiments were carried out with [³H]CP-55,940, Tris-binding buffer (50mM Tris·HCl, 50 mM Tris base; 0.1% BSA, pH 7.4), total assay volume 500 µl, using the filtration procedure described previously by Ross *et al.* [18]. Binding was initiated by the addition of mouse brain (CB₁ assay) or CHO cells (CB₂ assay) membranes (50 µg protein per tube). All assays were performed at 37 °C for 60 min before termination by addition of ice-cold Tris-binding buffer and vacuum filtration using a 12-well sampling manifold (Cell Harvester; Brandel) and Whatman GF/B glass-fibre filters that had been soaked in wash buffer at 4 °C for 24 h. Each reaction tube was washed five times with the wash buffer. The filters were oven-dried for 60 min and then placed in 5 ml of scintillation fluid. Radioactivity was quantified by liquid scintillation spectrometry. *Specific binding* was defined as the difference between the binding that occurred in the presence and absence of 1 µM unlabelled CP-55,940. The concentration of [³H]CP-55,940 used in our displacement assays was 0.7 nM. Each unlabelled cannabinoid tested was stored as a stock solution of 10 mM in DMSO, the vehicle concentration in all assay tubes being 0.1% DMSO. Protein assays were performed using a Bio-Rad Dc kit. The binding parameters for [³H]CP-55,940, determined by fitting data from saturation-binding experiments to a one-site saturation plot using GraphPad Prism, were 2336 fmol/mg protein (Bmax) and 2.31 nM (Kd) in mouse brain membranes, and 72570 fmol/mg protein (Bmax) and 4.3 nM (Kd) in hCB₂-transfected cells.

6.2.2. Intrinsic activity by in vitro assays.

Assays were performed according to the previously reported procedure based on P-ERK 1/2 expression evaluation in N1E-115 cells [23]. WIN 55,212-2 and rimonabant were adopted as CB₁ agonist and antagonist reference compounds, respectively. Tested and reference compounds were dissolved in Dulbecco's Phosphate Buffered Saline (DPBS) with 1% DMSO. Cells (European Collection of Cell Cultures, ECACC) were grown at 37 °C in humidified 5% CO₂ in Dulbecco's Modified Eagle's Medium e high glucose supplemented with 10% Fetal Bovine Serum (FBS), 1 mg/mL Penicilline Streptomycin, 2 mM L-Glutamine, 2.5 mg/mL Amphotericin B and 50 mg/mL Gentamicin. N1E-115 cells as well as the medium, the serum and all the supplementary additives were purchased from Sigma Aldrich (Milan, Italy).

For CB₁ agonism evaluation, cells were exposed to the compounds to be assayed or WIN 55,212-2 for 10 minutes (10 mL/mL of cell suspension). To determine CB₁ antagonism, assays were carried out adopting a 5 minutes of pre-treatment of N1E-115 cells with rimonabant or the compounds to be assayed, before the

exposure of the cells with the reference cannabinoid agonist WIN 55,212-2. In the case of the discovery of novel CB1 agonists, their intrinsic activity was confirmed through the capability of rimonabant to counteract the induced P-ERK 1/2 up-regulation. After the reported exposure time, all the samples were processed for western blot analysis according to previously reported procedures [23].

Protein concentrations was determined using Bradford Colorimetric assay (Bio-Rad) for western blot analysis. 40 µg/lane proteins were separated by 10% Bis-Tris Gel (NuPAGE® Novex® Invitrogen™) and transferred to nitrocellulose membrane (Bio-Rad). Blots were probed with specific Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (dilution 1:1000) and, after washing, with Anti-rabbit IgG, HRP-linked Antibody (dilution 1:3000). Chemiluminescence was visualised by Super Signal® West pico chemiluminescence substrate (Thermo Scientific). Immunoreactive bands were visualised by Fujifilm Las-1000 analyzer (Raytest Isotopenmessgeräte). The optical density of bands was measured using AIDA 2.11 software (Raytest Isotopenmessgeräte). Differences in P-ERK level were expressed as percentage of control ± SEM (five independent experiments). A one-way Anova followed by Dunnet's multiple comparison post test was performed for statistical analysing using GraphPad Prism 5 program (san Diego).

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Appendix. Supplementary material

Supplementary data related to this article can be trovato online at doi: These data include procedures for the preparation and analytical data of compounds **1a**, **1b**, **2a**, **2b**, and **14**.

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